MEMORANDUM

ON

ANAEMIA IN PREGNANCY IN INDIA

BY

L. EVERARD NAPIER, F.R.C.P. (Lond.)

AND

M. I. NEAL EDWARDS, M.D. (Lond.), W.M.S.

INCLUDING

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PREFACE

AT the annual meeting of the Scientific Advisory Board of the Indian Research Fund Association in December 1938, a sub-committee was constituted to examine and report on the results of the inquiries on anæmia in pregnancy which had been financed by the Indian Research Fund Association and to make recommendations for future work.

At its annual meeting in December 1939, the Board examined the report of the sub-committee as well as a summary, prepared by Dr. L. Everard Napier, of recent work in India on anæmia in pregnancy entitled 'Anæmia in Pregnancy in India: The Present Position'. The Board recommended that a memorandum on standard technique in regard to hæmatological methods and methods of survey should be prepared and published with Dr. Napier's summary and sent to the Central Advisory Board of Health with the object of drawing the attention of all Provincial Governments to the subject of anæmia.

These recommendations of the Scientific Advisory Board have now been carried out. Dr. Napier's summary is included in Part I of this publication, as well as a short chronological history of anæmia investigations under the Indian Research Fund Association from 1925 to 1939 and the report of the sub-committee appointed by the Scientific Advisory Board in December 1938. The memorandum on standard technique in regard to hæmatological methods has been prepared from a series of articles by Drs. L. Everard Napier and C. R. Das Gupta which were published in the *Indian Medical Gazette* between February 1940 and July 1941 and constitutes Part II, while instructions regarding methods of survey have been drawn up by Drs. M. I. Neal Edwards and L. Everard Napier, and are printed as Part III of this publication.

As an illustration of what can be accomplished by an anæmia inquiry conducted on sound lines, the summary and conclusions of the report on an anæmia inquiry conducted at Calcutta by Drs. Napier and Neal Edwards under the Indian Research Fund Association are given in Appendix I, not solely on account of the special importance of these conclusions, and certainly not because it is expected that similar conclusions will be arrived at from inquiries in other localities, but as an example of the type of conclusions that one can expect to draw from such an investigation.

Appendix II contains recommendations based on the findings of the Delhi Maternal Mortality Inquiry which was conducted by Drs. M. I. Neal Edwards and J. Dei under the Indian Research Fund Association.

Specimens of propaganda leaflets which were prepared during the Calcutta inquiry are given in Appendix III.

E. COTTER, LIEUT.-COL., I.M.S.,
Secretary, Governing Body and
Scientific Advisory Board,
Indian Research Fund Association.

SIMLA,
5th August, 1941.

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PART I

1

A short chronological history of anaemia investigations under the Indian Research Fund Association from 1925 to 1939

In 1925, Dr. Margaret Balfour, who had retired from the Women's Medical Service in India, visited India at her own expense with the object of carrying out investigations into the high maternal mortality in this country. She asked for a grant from the Indian Research Fund Association. In the report of the 3rd Research Workers' Conference held under the Association in 1925, it is stated that the services of a pathologist had been provided by the Women's Medical Service and that the grant asked for was for expenses of the inquiry only. A grant of Rs. 14,915 was recommended and was sanctioned by the Governing Body of the Association.

At the next conference (December 1926) the title of the investigation was extended and the anæmia of pregnancy was specifically mentioned. Dr. Balfour had drawn up and issued a questionnaire and had begun survey work in Bombay. The first publication on the subject appeared in September 1927 (*Indian Medical Gazette*) ; not much light was thrown on the ætiology, but the death rate in a series of 150 cases of anæmia was 42 per cent, and it was noted that the anæmia was hyperchromic in most cases.

At the 5th Research Workers' Conference in December 1927, the claims for an extension of this inquiry were stressed and as a result provision was made for additional staff and for the employment of a highly-trained worker from Europe. Dr. Lucy Wills was recruited from England and she started work in 1928-29. She was present at the next Research Workers' Conference (6th) ; in her report, she referred to vitamin-B deficiency as being the subject of some of her animal experiments. A separate inquiry on the effect of industrial conditions on maternal and infantile mortality was recommended.

At the 7th Research Workers' Conference (December 1929) Dr. Lucy Wills spoke on her work. She had separated the clinical picture of this form of anæmia which she claimed was different from Addisonian anæmia, or the secondary anæmia of malaria, hookworm and syphilis.

She had found some evidence of vitamin-A and vitamin-C deficiencies in her patients' dietaries and had commenced experimental work with these in animals. In the next budget provision was made for an investigation into still birth and

infantile mortality. During that year four reports appeared in the *Indian Journal of Medical Research* on these investigations.

At this same conference a grant was recommended for the investigation of hæmoglobin standards in health and disease in Bombay by Dr. V. R. Khanolkar, M.R.C.S. (Eng.), L.R.C.P. (Lond.).

At the 8th Research Workers' Conference (December 1930) Dr. Lucy Wills' resignation was announced. The ætiological importance of vitamin B was again suggested for investigation, but more especially in connection with still birth. Animal experiments were being carried out but no conclusive results were reported. It was during the next year that Dr. Lucy Wills published her historical observations on the effect of Marmite in tropical macrocytic anæmia (TMA), in the *British Medical Journal*, June 1931.

The scope of this inquiry showed signs of widening, and the Association financed inquiries on osteomalacia, maternity conditions in industrial workers, on early infantile mortality, and on still birth in India. Dr. Khanolkar reported his findings with different methods of hæmoglobin estimation.

At the 9th Research Workers' Conference (December 1931), the continuation of Dr. Khanolkar's inquiry was recommended. No results were given, or published, during the year. Other maternal mortality inquiries were discontinued.

A new inquiry was commenced by Dr. Mudaliar in Madras.

At the 10th Research Workers' Conference (December 1932), a report was presented on Dr. Khanolkar's inquiry.

Dr. Mudaliar sent in an extensive report on his lines of work but no results were given. He asked for a further year's inquiry with an increased grant of Rs. 6,000 which was recommended by the Scientific Advisory Board. A preliminary report on his work was published in the *Indian Journal of Medical Research*, October 1932.

It was at this meeting that Dr. Balfour made her appeal for an inquiry into maternal mortality in Assam where she had found an average of 42 maternal deaths per 1,000 live births, and in one district 137 per 1,000. She considered that half of the deaths were due to anæmia. She asked for a grant of Rs. 5,000. This sum was ear-marked for her inquiry which did not mature. The Assam Research Society issued a questionnaire with the help and advice of Dr. Balfour. Many replies were received but there is no evidence that the material was ever analysed.

At the 11th Research Workers' Conference (December 1933), Dr. Mudaliar's request for a grant of Rs. 5,840 for a scheme for future work was recommended.

At the 12th Research Workers' Conference (December 1934) Dr. Mudaliar's inquiry was regarded as completed. Dr. Napier put forward a proposal for work in Assam during 1935-36. This proposal was accepted. Dr. Bilimoria was transferred from Dr. Mudaliar's to Dr. Napier's inquiry.

At the Scientific Advisory Board meeting in that year a further sum of Rs. 4,200 was ear-marked for a survey of maternal mortality, but suitable proposals were not forthcoming.

At the 13th Research Workers' Conference (December 1935) there was considerable discussion on the necessity for more maternal mortality work and again a sum of Rs. 10,000 was ear-marked for surveys in maternal mortality in 1936-37.

Dr. Napier reported some preliminary results of his inquiry which was continued for a second year.

During the year 1936-37 a maternal mortality survey was commenced in Calcutta under Dr. Neal Edwards, W.M.S. A rural survey in Delhi was commenced but abandoned as it was not a success.

At the 14th Research Workers' Conference (December 1936), a maternal mortality sub-committee was formed; this committee recommended the expansion of the survey work, an inquiry on anæmia and eclampsia in Calcutta to take the place and to utilize the personnel of Dr. Neal Edward's survey inquiry, and the continuance of the anæmia inquiry in Assam. Dr. Napier gave a short résumé of his work which was being published in a series of articles in the *Indian Journal of Medical Research.*

At the 15th Research Workers' Conference (December 1937), the Maternal Mortality Committee again met and Dr. Neal Edwards gave a short report on her survey work which was completed and also on her anæmia investigations; these were incomplete and she thought they should be continued for another year. Dr. Napier agreed to take over this inquiry when Dr. Neal Edwards went on leave.

Dr. Napier reported the progress of his inquiry which was extended for another year. An inquiry on normal standards in women in Delhi was undertaken by Dr. Alice Benjamin.

At the 16th Research Workers' Conference (December 1938) the inquiry on anæmia amongst pregnant women was discussed and it was agreed that this inquiry should be closed down at the end of the year, as the work would be completed. Further reports on the work of Dr. Napier's inquiry had appeared in the *Indian Journal of Medical Research* during the year. This inquiry was continued. Dr. Alice Benjamin's report was received; this investigation appeared to be almost completed and was not continued.

2

Report of the Anaemia Sub-Committee appointed by the Scientific Advisory Board of the Indian Research Fund Association (December 1939)

Members:—Dr. M. I. Neal Edwards, Dr. V. R. Khanolkar, Lieut.-Colonel S. S. Sokhey, I.M.S., and Dr. L. E. Napier (Convenor)

The terms of reference for the Committee were: (1) to examine the results o the inquiries on anæmia, and especially those on anæmia in pregnancy financed by

the Indian Research Fund Association and (2) to make recommendations for future work on this subject.

Findings.—In India, anæmia *per se* presents a public health problem of primary importance. In the past it has attracted less attention than it deserved, partly on account of the general attitude of complacency that is adopted towards disease states not commonly associated with a high mortality, and partly on account of a physiological misconception, namely, that the normal hæmoglobin in the blood of persons living in tropical countries is lower than that of the residents of temperate climates. The misconception regarding the hæmoglobin level in the tropics has now been fully exposed as such, and whilst anæmia does not appear prominently in general mortality tables it is undoubtedly a very important factor in causing death in infectious and other diseases in which, had the patients started with full complements of blood and normal hæmopoietic systems, they would have recovered. Further, it is of outstanding importance as a direct or predisposing cause of maternal death.

Anæmia may be produced by a number of causes, but where it is widespread in a population, as it has been shown to be in a number of places in India, it is probable that it is due to a cause, or to causes, common to a large percentage of that population, though the dominant cause, or causes, of anæmia will be different in different localities: some of these causes are well recognized, whilst others are still obscure.

Anæmia assumes special importance in pregnancy; it is second only to sepsis as a cause of maternal deaths and it actually exceeds it in some parts of the country. The incidence of anæmia in pregnancy in various localities is probably correlated with the degree of anæmia in the general population—though this is a point about which more information needs to be obtained—but, while the conditions which produce the anæmia in the general population undoubtedly operate in the case of the pregnant woman also, it is probable that there is some additional ætiological factor.

The investigation of the problem of anæmia

Valuable indications of the ætiology of the anæmia in an individual can usually be obtained by studying the blood picture by modern hæmatological methods.

Hæmatological surveys in different localities where different conditions prevail have shown that there are a number of different causes for this widespread anæmia. In some instances the survey has clearly suggested what is the main cause of the anæmia, and such surveys, carried out in a large number of places where other conditions prevail, *if the results are correlated with the local conditions, especially the local dietary and prevalent infections*, will add to our knowledge regarding the characteristic blood pictures associated with various sets of conditions, as well as giving valuable indications as to the cause of the anæmia in any of these places in which anæmia is found to be prevalent, and thereby will demonstrate the ways by which it may be controlled.

Hæmatological surveys should include:—

(1) Clinical examinations and hæmoglobin estimations to find out the incidence of frank anæmia in the population;

(2) A fuller blood examination in a sample of so-called normal individuals;

(3) A complete blood examination in a group of frankly anæmic individuals (which examination, if possible, should be repeated during and after treatment);

(4) A complete blood examination, with epidemiological and clinical data, in two series of pregnant women, non-anæmic and anæmic. (This examination should be repeated later during pregnancy and after delivery.)

The provision of specially trained workers

It is essential in hæmatological work of this kind that, as data which are unreliable are worse than useless, a high degree of accuracy should be maintained, which can only be expected from trained personnel. The Indian Research Fund Association cannot of course be expected to undertake work over such a wide field as has been visualized above, but as far as possible workers who have already been trained in this work should be retained and should be utilized to carry out such surveys, by themselves, or in centres where they can supervise the work of other personnel and instruct them in modern hæmatological methods, or in a central laboratory where suitable individuals could be sent for training in this work.

Recommendations

(*a*) It is recommended that *hæmatological surveys*, on the lines suggested above, should be carried out, both in urban and rural areas.

It should be possible in many instances for these hæmatological surveys to be carried out by members of the staff of hospitals and colleges, and the surveys in rural areas might provide a good opportunity for short-term inquiries by university scholars, after preliminary training at some central laboratory where this type of work is done.

(*b*) Although it is not recommended that, at the present time, experimental work be extended beyond the experiments actually visualized, from time to time specific problems which require, and are suitable subjects for, experimental confirmation are likely to arise and for this purpose trained personnel will be essential.

(*c*) To ensure uniformity, in both technique and in the general procedure in the hæmatological surveys, so that the findings in different localities may be comparable, it is recommended that a *memorandum* be issued in which details

of hæmatological methods and suggestions for the conduct of hæmatological surveys, in both urban and rural areas, are given.

(*d*) It is further recommended that in conjunction with this memorandum (or in a separate publication) a summary of recent work done in India on this subject, including suggestions for future lines of work, a draft of which has already been prepared, should be issued.

3

Anaemia in pregnancy in India: The present position

About thirteen years ago, special attention was drawn to the importance of anæmia as a cause of maternal morbidity and of maternal and infantile mortality in India. During the intervening years a considerable amount of investigation has been undertaken both under the auspices of the Indian Research Fund Association and by independent workers. We feel that the time has now come to summarize the results of this work, to state the present position, and to indicate the general trend of these investigations.

Maternal mortality in India

The exceptionally high maternal mortality in child-birth in India may be looked upon as the circumstance that made an inquiry on this subject an urgent necessity.

Dr. Margaret Balfour who had retired from the Women's Medical Service returned to this country at her own expense to investigate this subject. She received a grant for additional help from the Indian Research Fund Association.

She (Balfour, 1927*b*) collected data from Indian hospitals and found a maternal mortality rate of 21·5 per mille (244 deaths in 11,343 deliveries).

The registration of both births and deaths in India generally is unsatisfactory, and, as there is no special mechanism by which deaths associated with child-bearing are returned separately, the central or provincial public health reports give no indication of the true state of affairs.

In Bombay, however, where it was thought that the registration was comparatively accurate, the death rate was given as 16·7 per mille births in 1924 and in the Calcutta Corporation Health Officer's report for the same year it was given as 18 per mille.

In 1933, Sir John Megaw collected figures from rural dispensary doctors in different parts of India and gives a figure of 24·5 maternal deaths per 1,000 live births. In a special inquiry in Calcutta, Neal Edwards (1939) found a similar rate

and two inquiries in Madras obtained figures of 16·6 and 18·5 per 1,000 births. In Assam, as a result of a questionnaire, Balfour obtained an average figure of 42 per 1,000, and in a single area the extraordinary figure of 137 maternal deaths per 1,000 live births. These figures have to be compared with 3·8 and 3·16 per 1,000 live births in England and Wales in 1936 and 1937, respectively. It is unnecessary to emphasize this point further.

The part played by anæmia in this high mortality

Balfour in her early investigations and personal experience in Bombay and Assam repeatedly expressed the opinion that about half the deaths were associated with anæmia.

She (Balfour, 1927*a*) placed anæmia second to osteomalacia as a cause of maternal morbidity and gave the figure as 27·6 per mille of pregnancies. Neal Edwards collected data from women's hospitals in India and found even higher figures for anæmia, 49·5 and 42·9 per thousand in 1936 and 1935, respectively. Napier and Das Gupta (1937*b*) found 158 per thousand pregnant coolie women in Assam definitely anæmic (below 50 per cent). Choudhury and Mangalik (1938) found 5·6 per cent of pregnant women in Agra 'anæmic'; in their published series of 41 cases they include two with 50 per cent hæmoglobin (6·87 g.) and seven over this amount.

Balfour (1927*b*) reported that 61·9 per cent of all maternal deaths in Bombay and 35·6 per cent of those from India generally were due to anæmia.

Mudaliar and Rao (1932) in Madras city found that 50 out of 436, or 11·5 per cent, of maternal deaths were due to anæmia, but Neal Edwards in her Calcutta inquiry (1936-37) found that 23·33 per cent of deaths were due to anæmia and from an analysis of 39 women's hospitals all over India, 18·0 per cent. Mitra (1933) gives the mortality due to anæmia during 1931 at a large maternity hospital in Calcutta as 47·9 per cent of the total maternal mortality.

The death rate in pregnancy anæmia.—The significance of the figures quoted below is limited, as the death rate will depend, firstly, on the severity of the cases admitted and, secondly, on the treatment given.

Number of patients	Percentage of deaths	Authority
150	42	Balfour (1927*a*).
43	34·9	McSwiney (1927).
50	30	Wills and Mehta (1930*a*).
165	31·5	Mitra (1937).
561	36·5	Mitra (1937).

These figures do however show that the maternal mortality in pregnancy anæmia may be alarmingly high. Napier and Majumdar (1938) had eight deaths in a series of 58 cases (13·8 per cent), but there were in this series seven additional cases noted as 'failures' and it is probable that some of these patients died later in their homes. It is possible that a similar explanation may be given for Chatterjee's (1938) low figure—12 per cent.

An even higher infantile mortality is reported by all workers; for example. McSwiney (*loc. cit.*) gives a figure of 58·8 per cent.

Large as the figures are, they do not convey a true picture of the effect of anæmia, because amongst the cases of sepsis, which heads the list of causes of maternal death, there are many instances in which, if the patients had not been severely anæmic as well, they would have recovered.

Whereas, in Bombay and Calcutta, sepsis and eclampsia which appear above and below anæmia as the first and third causes of death, respectively, occur in all countries, anæmia is usually classed as a negligible factor in Western countries, accounting for only 0·05 (England and Wales) and 0·08 (Scotland) per cent of maternal deaths. Anæmia is therefore not only very important as a cause of maternal mortality, but it is an outstanding one in this country as compared with other countries.

Further points which might be ascertained are the comparative importance of anæmia in Northern India and in any place where hookworm and/or malaria are uncommon.

Epidemiological data

The early papers on the anæmia of pregnancy discuss the epidemiology, but usually give impressions rather than figures, and such figures as are given seldom include the composition of the populations and are thus valueless. Balfour (1927*a*), however, shows that in Bombay anæmia in pregnancy is more common in Mohammedans than in Hindus. The incidence per 1,000 is 93·8 and 40·1, respectively. However, osteomalacia and eclampsia, two other diseases which she includes in her table, show a far greater preponderance amongst Mohammedans, which suggest that the community is unhealthier generally. She states that they live on a better diet than the Hindus, but does not give any data for this observation.

Gupta (1932), reporting maternity cases admitted into the Eden Hospital, Calcutta, during 1928 to 1930, gave the total incidence of anæmia as 7·19 per cent; Hindus were 8·89 per cent, Mohammedans 19·09 per cent, and Indian Christians 22·54 per cent, the low total incidence being due to dilution with Europeans, none of whom were anæmic of 73 admitted, and with Anglo-Indians in whom the incidence was also low.

Choudhury and Mangalik (*loc. cit.*) found little difference in the communities in Agra: they give Hindus as 5·1 per cent and Mohammedans as 6·3 per cent.

There are very few data regarding the economic status of patients. Both Balfour and Wills repeatedly state that anæmia of pregnancy is not confined to

the poorer classes and that it is higher amongst some of the better-class communities. They produce no convincing figures to prove this. On the other hand, most of the data presented are from hospitals in which the poorer class of patient predominates, and, though anæmia is known to be far from uncommon amongst the 'private-patient' class, comparable figures are not available. As far as Bombay is concerned the statement is probably dependent largely on the observation that anæmia in pregnancy is not common amongst a certain class of mill worker. The dietary surveys in Bombay (Wills and Talpade, 1930; Talpade, 1931) amongst different classes of patient, including the mill workers, produced mainly evidence of a negative nature, in that they showed that the individuals of all the classes surveyed lived on an ill-balanced diet; the diet amongst the mill workers was very deficient in fat and protein, particularly animal protein, and in vitamins A and C, but was not deficient in vitamin B; this did not, however, differ significantly from that of the pregnant anæmics of the hospital class.

Seasonal incidence.—Balfour (1927*a*) in Bombay found the highest incidence from September to March, McSwiney (*loc. cit.*) and Mitra (1931, 1937) from July to December in Calcutta (*see* Table I).

TABLE I

	Balfour (1927*a*) (percentage)	Mitra (1931)	McSwiney (1927)
January ..	10	4	3
February ..	7	2	2
March ..	13	1	1
April ..	5	5	0
May ..	2	3	0
June ..	1	6	4
July ..	5	8	5
August ..	6	6	3
September ..	10	11	7
October ..	9	12	3
November ..	14	10	7
December ..	18	18	8
TOTALS ..	100	86	43

Chatterjee (*loc. cit.*) gives the percentage incidence of pregnancy anæmia compared with total obstetric admissions at a Calcutta hospital over a 5-year period; he shows that there is a marked preponderance from October to January with comparatively high percentages in September and February.

Mudaliar and Rao (*loc. cit.*) in Madras were only reporting on a small number but show the highest incidence in June. Choudhury and Mangalik (*loc. cit.*) in Agra do not find any seasonal incidence. They point out that the pregnancy incidence increases in the second half of the year. This was shown also by Balfour in Bombay and is true in Calcutta, but in neither city is this increase in the anæmia incidence in the last four months of the year explainable on the greater number of deliveries in these months. Balfour found diarrhœa more common in the second half of the year and considers that the anæmia incidence is correlated with this.

Age.—The question of age is too closely associated with that of parity to provide any significant information independently.

Parity.—Balfour (1927*a*), McSwiney (*loc. cit.*), and Chatterjee (*loc. cit.*) give the following figures for parity (Table II):—

TABLE II

Authority:—	Balfour (1927*a*)	McSwiney (1927)	Chatterjee (1938)
Locality:—	Bombay	Calcutta	Calcutta
Primiparæ ..	29	13	10
2nd pregnancy ..	22	10	7
3rd „ ..	13	6	10
4th „ ..	7	5	9
5th „ ..	9	2	7
6th „ ..	3	3	3
7th, 8th, or over ...	14	2	3
Totals ..	97	43	50

Mitra (1937) and Choudhury and Maugalik (*loc. cit.*) show graphs suggesting the same steadily declining incidence through the pregnancies. The value of these figures is reduced by the writers' failures to give the parity distribution amongst normal pregnancies. This will obviously vary in different populations. Mitra (1937) goes further and makes the misleading statement: 'Figure 2 shows the greater incidence in multiparæ (80 per cent) than in primiparæ.' Napier and Gupta (1937*a*) remedy this defect, but they are obviously dealing with an unusual population for the peak of the normal distribution curve is at the third pregnancy: in their figures the percentage of anæmia is 28·5 for primiparæ and this falls to 15·4, 13·3 and 12·2 in the succeeding pregnancies.

From the data analysed there appears to be evidence of a higher relative incidence of anæmia amongst primiparæ, but it is possible that in some instances this apparent predominance is due to the fact that women are more likely to come into hospital for their first pregnancy.

Prematurity.—Not very much is to be learnt from the month of pregnancy at which the patient first comes under observation as this may have little relationship to the time of onset of the anæmia. In nearly all the series reported, the incidence of anæmia rises towards the end of pregnancy, and very few cases are less than six months pregnant when they first come under observation.

Discussion.—Studies of the anæmia of pregnancy in which an attempt is made to find epidemiological factors are usually based on the premise that there is one specific condition with one common cause. Discrepancies in the findings of different observers may well be explained on the grounds that there are multiple causes and that these are not equally represented in the various series of different observers. Discrepancies therefore assume a special importance and, further, it is obvious that we ought to obtain more data from different places in India and from different classes of patients, in order to decide more definitely whether the cause is single or multiple, and to correlate the various observations with the special local conditions, e.g. the incidence of various diseases, such as hookworm and malaria, and with the diet of the populations concerned.

Associated clinical picture

Wills and Mehta (1930*a*) have stated that 16 of their 66 cases have been excluded on the grounds of malaria, hookworm infection, syphilis, or definite nephritis. The rest they claim are 'idiopathic' anæmia of pregnancy. This procedure of excluding certain cases seems scarcely justifiable and only likely to confuse the issue.

Fever.—This is a very common associated condition. Balfour (1927*a*) noted that the onset of the anæmia was with fever in exactly half her cases and that 83·3 per cent had fever at some time or another during the disease. Mitra (1931) reported fever in 36·7 per cent of cases, and Chatterjee (*loc. cit.*) in 90·6 per cent. Other workers refer to the frequency of fever. Napier found in his Assam cases that fever at some time during pregnancy was the rule in almost all cases.

Little importance can be attached to the percentage malarial parasite findings in the various series reported, as much will depend on the thoroughness of the search made. Balfour (1927*a*) reported 8·66 per cent plasmodium infections in her series, a high figure in a place where the general malarial incidence is not particularly high.

Splenic enlargement.—In Bombay. Balfour (1927*a*) reports 18 per cent splenic enlargement. McSwiney (*loc. cit.*) 23 per cent, Mitra (1931) 40·7 per cent, and Gupta (*loc. cit.*) 8·8 per cent in Calcutta; the last is probably too low a figure. In Assam, Napier and Bilimoria (1937) differentiated between the hæmatological groups; they reported 33 per cent enlarged spleens amongst hyperchromic cases, 14 per cent amongst hypochromic, and 6 per cent amongst non-anæmic pregnant women. Napier and Majumdar (*loc. cit.*), in a more malarious part of Assam, found 65 per cent of the anæmic pregnant women had enlarged spleens, against 30 per cent non-anæmic, if all degrees of enlargement were considered, and 43 per cent and 8 per cent, respectively, if definite measurable enlargement only was considered; they also found that there was a 'significant' difference between the iron-reacting and the liver-reacting groups, the latter showing a much higher incidence of splenic enlargement.

In Agra, Choudhury and Mangalik (*loc. cit.*) found that 55·6 per cent of the patients in their macrocytic group had enlarged spleens, but none of the rest.

All reports thus tend to emphasize a very high incidence of splenic hypertrophy amongst cases of anæmia in pregnancy.

Œdema.—All workers report a high incidence of œdema and many say it is a cent per cent finding.

Albumin in the urine.—Most workers report this in a large percentage of cases.

Wills and Mehta (1930*a*) report albumin in the urine in 35 per cent of their cases, Balfour (1927*a*) in 48·6 per cent, McSwiney (*loc. cit.*) in 60·5 per cent, Mitra (1931) 26 per cent, and Gupta (*loc. cit.*) 25 per cent, but Chatterjee (*loc. cit.*) only gives 7·5 per cent; this last figure can probably be ignored.

Gastro-intestinal symptoms.—Diarrhœa is a very common association; it was reported in 38 per cent of Balfour's (1927*a*) series, in 44 per cent and 20·6 per cent of Mitra's (1931, 1937) two series, and in 74·1 per cent of the macrocytic group in Choudhury and Mangalik's (*loc. cit.*) series. In the Assam cases diarrhœa or dysentery occurred in at least two-thirds of the cases either shortly before or during the time that they were under observation. Balfour was so impressed with the frequency of the association that she attributed the higher incidence of anæmia in the second half of the year to the much higher incidence of diarrhœa and dysentery in Bombay at this season.

Balfour (1927*a*) reported vomiting (other than the ordinary vomiting associated with early pregnancy) in 40 per cent and sore tongue in 31 per cent of cases. Mitra (1931) mentions that 82·5 per cent of his series had a sore tongue. We have

found in Calcutta and Assam this condition very hard to assess; most of the women eat *pan* which disguises the appearance of the mouth and most of them have pyorrhœa, and it is difficult to elicit the information without a leading question; our impression is that it is not a very prominent symptom. Choudhury and Mangalik (*loc. cit.*) state definitely that they have not noted it in their cases. Others do not mention either vomiting or sore mouth as common.

Gastric acidity is discussed later.

Wassermann and Kahn reactions.—McSwiney (*loc. cit.*) found a positive Wassermann reaction in 40 per cent of his cases. Balfour (1927*a*) reported that, in 15 out of 32 cases tested, the Kahn reaction was positive. Mitra (1931) found four out of 11 cases Wassermann positive; Napier and Majumdar (*loc. cit.*) found the Wassermann reaction negative in 27 out of 36 cases, doubtful in three, and positive in six; most of the positive findings were in the hypochromic group. Chatterjee (*loc. cit.*) however found *none* of his cases positive, and other workers have not found more positives than the usual percentage of the general population. This subject requires further investigation as no large series has been done.

Treatment.—No one has described the treatment of anæmia in pregnancy with much confidence and those who have attempted any scientific appraisal of their results have usually approached the subject with the object of throwing light on the ætiology.

McSwiney (*loc. cit.*) advised treatment on general hygienic lines, with dietary, iron and arsenic by mouth or injection, small injections of whole blood, and the termination of pregnancy. He advised against blood transfusions.

Green-Armytage (1928) thought that the anæmia of pregnancy was a toxæmia and advocated the termination of pregnancy.

Wills and Mehta (1930*a*) used liver extract with good results.

Wills (1931) first drew attention to the value of Marmite. In a large number of her Bombay cases recovery followed the administration of 30 grammes of Marmite daily. The improvement occurred after a typical reticulocyte response.

Gupta (*loc. cit.*) recommended treatment on similar lines but included liver injections and Marmite. He noted that large transfusions caused abortion.

Mudaliar and Rao (*loc. cit.*) obtained no response with Marmite. This is possibly because many of their cases were hypochromic and probably iron-deficient; liver extract and iron produced the best response.

Mitra (1937) advocated all known forms of therapy but was vague as to the results obtained. He reports a high mortality. He seems to favour small repeated transfusions, but his remarks suggest theory rather than practice. He attributes good results to intravenous glucose. He concluded 'Cure is never vouchsafed unless pregnancy terminates'.

Choudhury and Mangalik (*loc. cit.*) also found Marmite useless. They obtained good results with Neo-Hepatex and Campolon in macrocytic cases, and with

iron in microcytic. Transfusion was not used by them as they considered it unnecessary.

In Napier and Majumdar's (*loc. cit.*) series, treatment was experimental and usually one hæmatinic only was given :—

Iron (in microcytic cases) or Marmite (in normocytic cases) given early seemed to be effective, but given in the later stages of pregnancy it had no effect until the uterus was empty. Campolon produced a more rapid, and a more certain, response in normo- or macrocytic cases, but again the inhibitory effect of the fœtus was marked, and, in the later stages of pregnancy, response was seldom satisfactory until the uterus was empty.

Transfusion seemed to have no specific effect but tended to cause abortion which was often followed by improvement.

Napier *et al.* (1938) found Anahæmin less effective than crude liver extract. This observation was confirmed by Wills and Evans (1938).

Hæmatological investigations

The necessity for investigating the hæmatological picture of anæmia of pregnancy led to work in a number of divergent directions, which it will be necessary to consider separately. The first may be called the introduction and standardization of hæmatological methods and the obtaining of normal standards for Indian populations. The second is the attempt to separate into different hæmatological groups, in the hope that this may lead to an ætiological classification, the anæmias that occur (*a*) amongst the general population, both men and women, and (*b*) in pregnant women. A third direction has been the evolution and study of a new pathological and clinical syndrome, tropical macrocytic anæmia.

It will be convenient to discuss the last line of investigation first.

Tropical macrocytic anæmia.—Wills in her first paper showed very clearly that there was amongst her cases of anæmia of pregnancy a hyperchromic anæmia which was quite distinct from Addisonian pernicious anæmia, but which responded with a characteristic reticulocytosis to liver extract; she also described a similar disease in non-pregnant women. Her biochemical and clinical investigations in these cases did not suggest that the condition was a toxæmia of pregnancy, and on the grounds of some preliminary dietary investigations amongst her patients she suggested that it was most probably a dietary disease and she favoured deficiency of vitamins A and C, as being the probable cause (Wills and Mehta, 1930*a*).

A dietary survey amongst different Bombay communities seemed to add support to the suggestion that vitamin-A and vitamin-C deficiencies were the causative factors, as it was in the communities in which these deficiencies were most pronounced that most cases of this anæmia were discovered (Wills and Talpade, *loc. cit.*). More support came from animal experiments in which she

showed that rats fed on diets deficient in vitamins A and C died of *Bartonella* anæmia (Wills and Mehta, 1930*b*). She then turned to monkeys as her experimental animals. She produced a macrocytic anæmia in monkeys by feeding them on a diet of polished rice, white bread, and chapattis, a more-or-less vitamin-free diet; to this she added vitamins A and C but without result; she then gave Marmite, with the result that the monkey's red cell count and its weight immediately improved (Wills and Bilimoria, 1932). She followed this up by an immediate clinical trial in Bombay and established the fact that Marmite would cure one form of anæmia of pregnancy, a macrocytic anæmia, that appeared to be most prevalent in Bombay (Wills, 1931).

This work of Wills had clearly established the fact that there was a macrocytic anæmia occurring amongst pregnant and non-pregnant women which was curable by some substance contained in autolysed yeast; this disease which was a new syndrome she called tropical macrocytic anæmia.

The subsequent work of Wills (1934) has not been done under the ægis of the Indian Research Fund Association; it is however important work and some of it has been done in India. Wills and her co-workers have shown that the actual substance that produces the improvement in this macrocytic anæmia is not vitamin B_1, lactoflavin, nicotinic acid, vitamin B_4, or vitamin B_6. She showed that, in experimental monkeys fed on a diet deficient in vitamin-B complex, the West and Dakin fraction of liver extract (represented by Anahæmin) was not curative, but that the soluble fraction, after saturation with ammonium sulphate, of Campolon produced a reticulocytosis and cure. Napier (1938) confirmed this in the case of tropical macrocytic anæmia in Calcutta as did Wills and Evans (*loc. cit.*) in Bombay a few months later. Later, however, the former worker showed that in some cases a cure was effected with massive doses of Anahæmin, in conjunction with a good hospital diet (Napier, 1939*b*). Later, Foy and Kondi (1939) made the same observation with regard to macrocytic anæmia in Macedonia.

The present position is that the actual identity of the substance that brings about a cure in Wills' macrocytic anæmia has not been discovered, but a large number of substances have been excluded; to those substances mentioned above must be added Castle's extrinsic factor, as refined liver extracts (e.g. Anahæmin) which are curative in true pernicious anæmia are not as effective in this condition. We know that the substance is present in an ordinary mixed diet, and in a more concentrated form in autolysed yeast and in the cruder liver extracts.

In the experimental work with monkeys at present being undertaken by the writer with the assistance of Dr. Majumdar, we have so far failed to reproduce the Wills' macrocytic anæmia in our monkeys, though we have been able to show that the recovery time in monkeys subjected to repeated small bleedings was much longer when the monkeys were kept on a vitamin-B-deficient diet than when they were kept on a balanced diet.

Whilst it is a matter of more than academic interest to identify this factor, the writer does not feel that it is an absolutely essential piece of information, not, at any rate, to the extent that lack of this knowledge seriously prevents our obtaining

a very much better understanding of the ætiology of the anæmias of pregnancy in India. We know that a diet deficient in vitamin-B complex will probably be deficient in this substance and tend to produce a particular type of macrocytic anæmia. and we know that autolysed yeast and most of the crude liver extracts contain it and, in uncomplicated cases of this anæmia, will effect a cure. The writer does not think that at this stage further experimental work in India directed solely towards identifying this factor will be profitable. It is highly technical work which is already being done in England by Wills and probably other workers. Later, it might be necessary to confirm her findings in India as we did in the case of her Anahæmin work.

We know that deficiency of a certain food factor will produce this particular macrocytic anæmia. We know also that many of the people who suffer from it live on a diet which is deficient in many essential substances, but none of the dietetic survey work so far done, either in Bombay or elsewhere, has been able to show that the diets associated with this anæmia were definitely deficient in vitamin-B complex, though it was seldom in excess. Wills has taken the view that the pregnant women's requirements are slightly in excess of those of a non-pregnant woman and that this deficiency is thereby exaggerated; she points out quite correctly that this same anæmia occurs in non-pregnant women and in men, but less frequently. In this connection the example of iron deficiency and hypochromic iron-deficiency anæmia in pregnant women has been quoted. However, it has not been shown that the demands of the fœtus for vitamin-B complex are greater than for any other food substance, as is the case with iron which the fœtus stores in its liver in large quantities. All workers are not prepared to accept this comparatively simple explanation of the ætiology of the syndrome. Reference will be made to this point later.

Tropical macrocytic anæmia hæmolytica.—Napier (in 1936) drew attention to the fact that all macrocytic anæmias of tropical countries that responded to Marmite and liver extract were not Wills' tropical macrocytic anæmia; he pointed out that there was a hæmolytic group, associated with hyperbilirubinæmia, a high reticulocyte count, and usually splenic enlargement, possibly of malarial origin. Fairley and his co-workers (1938) found the same state of affairs in Macedonia; Fairley considered that there were two forms of tropical macrocytic anæmia, a hæmolytic and a non-hæmolytic. Napier (1939*b*) developed this idea further; he concluded that there was strong evidence in favour of the existence of a hæmopoietic principle, distinct from the hæmopoietic principle absent in pernicious anæmia and in no way associated with gastric dysfunction, which substance was essential to the proper maturation of the red cells; it was a substance that was not synthesized in the body and was not a residue of normal hæmolysis, but had to be provided in the food; its deficiency might be actual, that is, a true food deficiency, or relative, that is, due to the extra demands of excessive hæmolysis (e.g. of post-malarial reticulo-endothelial tissue hypertrophy), or due to mal-absorption in, for example, long-continued diarrhœa.

These observations of Napier and Fairley and their co-workers have not so much introduced a new clinical and pathological syndrome, as they have broadened

the basis of Wills' syndrome, so that it includes more than one type of anæmia, and they have certainly widened the ætiological possibilities.

Normal hæmatological standards for Indian populations

One of the first difficulties encountered by anyone attempting to work on anæmias in India ten years ago was the complete lack of any normal standards for Indian populations, and the primitive state of hæmatology in India. At this time most of the large hospitals in the country depended on the Tallqvist scale for the estimation of hæmoglobin; this and the differential count were practically the only routine hæmatological investigations ever carried out. This lack of precision in hæmatological methods was not confined to India and the fact that the 100 per cent mark in some hæmoglobinometers indicated a hæmoglobin content of 13·8 grammes in 100 c.c. of blood, whereas in others it corresponded to 17·2 grammes showed that hæmatology throughout the world was in a very confused state at this time.

At one meeting of medical officers that was called in Assam to discuss with Dr. Balfour her proposals for investigating the anæmia of pregnancy, a great deal of time was spent on discussing whether 60 per cent or 65 per cent should be looked upon as the normal for Indian women; no consideration was given to the scale or the method of measurement used, which was of course usually the Tallqvist. This is not quoted as a criticism of the medical officers present, but to demonstrate the confused thinking that was prevalent at the time.

At the 7th Research Workers' Conference, Mehta made a strong plea for the collection of hæmatological data from normal Indian populations. A very important achievement of the investigations under the Indian Research Fund Association has been the stimulation of work of this kind, much of which has actually been done by officers employed by the association. Papers in which the different hæmatological methods were critically examined were written by Sokhey, Napier, and others, and have undoubtedly helped considerably in uniformalizing hæmatological methods in India. Some of the findings amongst normal populations are given here.

Normal hæmatological data

In the three tables below some of the important data on hæmatological standards for Indian populations have been collected. In the United States and in Great Britain much work on normal standards has been done; we have quoted only representative data from reliable authorities*.

Red cells.—There is a striking uniformity in the findings. The average count for males in Western countries is 5·5 millions, in Indian towns 5·4 millions, and in coolie populations 5·3 millions. For females, the average for Western countries is 4·8 millions and for Indian populations 4·5 millions.

* These tables will be found on pages 41, 54 and 67.

A series by Basu and Chatterjee (1937) have been excluded as they are so grossly at variance with the findings of other workers; they give, for a series of women in Calcutta, 3·6 millions with a mean deviation (*sic*) of 0·49 million.

Hæmoglobin.—There is less uniformity in the hæmoglobin estimations; for men the means given range from 14·5 grammes to 16 grammes in Western countries (higher and lower readings are given by other authorities not quoted here) and those for men in Indian towns cover about the same range. On the other hand, the figures obtained amongst coolie populations are markedly lower (12 to 13 grammes) and show high coefficients of variation. Similarly amongst the women 13·5 grammes is about the average for Western countries and 13 grammes for Indian towns, against 10·5 grammes for coolie women.

Corpuscular values.—This constancy in the red cell counts and deficieney in the hæmoglobin levels amongst coolies is naturally reflected in the corpuscular values, and both the mean corpuscular volume (MCV) and the mean corpuscular hæmoglobin (MCH) of the cells of the coolie populations is in each case much lower than that of the town dwellers. It will be noted that the MCV of the two Cachar series were not nearly as low as those of the two Assam series; it will be noted also that the mean corpuscular hæmoglobin concentration (MCHC), (which is complementary to the MCV, if the MCH is constant) is exceptionally low in the former series. These two interdependent observations can probably be accounted for by the unsatisfactory working of the centrifuge which was noted in the report on the Cachar series.

Hyperbilirubinæmia.—The van den Bergh test was not done in many of the 'normal' series; the assumption was that it would be 'negative'. Vaughan and Hazlewood (1938) did this test in a series of 'normal' individuals in England and obtained the results shown in Table III. Data from Assam coolies are also shown:—

TABLE III

Van den Bergh indirect

Locality	Number of persons	0·5 mg. or less per 100 c.c.	Less than 1·0 mg. but over 0·5	1·0 mg. or over	Percentage over 1·0 mg.	Authority
England ..	100	64	32	6	*6*	Vaughan and Hazlewood (1938).
Cachar ..	50	39	6	5	*10*	Napier and Majumdar (1938).
Assam ..	40	33	3	4	*10*	Napier and Bilimoria (1937).

Vaughan and Hazlewood find a distinctly higher percentage of positives than many other English and American observers, but they are not as high as those amongst so-called normal individuals in Assam.

Reticulocytes.—The normal figure is about 0·5 per cent of the total red cells: some writers give a lower figure. From the Indian 'normals', the following data are quoted (Table IV):—

TABLE IV

Reticulocytes

Locality	Material	Number of persons	Percentage	Standard deviation	Authority
Calcutta ..	Men	50	0·67	± 0·37	Napier and Das Gupta (1935*b*).
„ ..	Women	122	0·37	± 0·27	Napier (1939*a*).
Assam ..	Men and women	41	2·17	± 1·92	Napier and Das Gupta (1935*c*).
„ ..	Pregnant women	40	2·10	+ 1·80	Napier and Bilimoria (1937).

This suggests that the normal reticulocyte count in Indians is the same as that in Europeans, and it seems probable that in the Assam series the high reticulocyte count is evidence of disturbed erythropoiesis.

Red cell diameters.—The only reliable way of learning the mean diameter of red cells is by Price-Jones' method, or some slight modification of it. A few series have been done on normal Indians. Maplestone (Chaudhuri, 1933) found the mean diameter of 500 cells of each of ten healthy Indians to be 7·270μ. Sankaran and Radhakrishna Rao (1938) found a curiously low figure for Madrassi Indians; the mean of about 500 cells from each of 25 normal Indians, male and female, was 6·85μ ± 0·28μ; there was no significant difference between males and females in this series. Napier *et al.* on the other hand obtained a figure of 7·288μ ± 0·468 for 50 Bengal males. Price-Jones' figure was 7·202μ ± 0·172μ for 100 healthy individuals in England.

The low figure obtained by Sankaran and Radhakrishna Rao is not explained but otherwise there seemed little indication that the measurements differ materially from those obtained elsewhere; Price-Jones' figure is representative of the European findings. The much wider range of variation amongst the so-called normals probably indicates that strict normality is less common in Calcutta than in London.

Discussion.—These data all conform to the conclusion that, as far as the red cell series is concerned, there is no physiological difference in the blood pictures between Europeans in temperate countries and Indians in India, and that differences, when they occur, are due to some pathological cause; this cause may be anything from a food deficiency to a sub-clinical infection. In the case of the coolie populations, there is in the hæmatological data further evidence to support this suggestion, in the high coefficient of variation in the hæmoglobin estimations, in

the high mean reticulocyte count, and in the hyperbilirubinæmia that are commonly encountered amongst Assam coolies.

The question naturally arises as to the reason for this lower level of hæmoglobin amongst the Assam coolie populations and whether it can be traced to the special conditions prevailing in Assam. The only figures we can quote from our data are those from Shivrajpur coolies; these are economically comparable, but are employed in a different part of the country. The hæmoglobin means are much closer to those of the Assam coolies than to those of the city-dwelling Indians. This suggests that the hæmoglobin deficiency is one associated with economic status and dietary rather than with the particular local conditions. It has been our experience in Calcutta, as well as in Assam, that not only has the average healthy ryot a lower hæmoglobin level, but that his cells tend to be smaller and, though long-continued treatment with iron will bring his red cells up to the 5·5 millions level or even higher and his MCHC up to 35 per cent, on account of the persistently small size of his red cells his hæmoglobin will not rise to the normal level

Napier and Das Gupta (1936) did a small experiment in this connection; they gave a course of iron treatment to six healthy coolies, and only managed to raise the mean hæmoglobin level from 11·2 grammes to 12·0 grammes. This is certainly an experiment that should be repeated, but the implication of this very poor response to iron therapy is that there is some other factor operating which keeps down the size of the red cell and consequently the hæmoglobin level.

It will obviously be easier to answer this question when we have more data. We want 'normal' data for village populations in different parts of India where different conditions prevail. This is an important point, for one may safely conclude that there will be some connection between the general low level of hæmoglobin and the incidence of gross anæmia in any population, even if the cause is not exactly the same. Meanwhile, any further consideration of this aspect of the problem can be postponed until some of the possible causes of the anæmia are discussed.

The blood picture in pregnant women

Dieckmann and Wegner (1934) and Bethel (1936) have found that there is a progressive increase in blood volume throughout pregnancy. This is not accompanied by a corresponding increase in cells, so that there is a relative decrease in the number of cells in a fixed volume of blood—this physiological anæmia amounts to about a 14-per cent deficiency, i.e. 14 per cent below the normal level for non-pregnant women. In order to make allowances for the natural variations in individual hæmoglobin percentages these writers allow a further margin, and state that any reading below 10 grammes should be considered as indicating anæmia —this figure is about 27 per cent below Bethel's mean for non-pregnant women (13·78 grammes). Translating this into terms of Indian populations, we get about 9·5 grammes for town and 7·7 grammes for coolie populations on the 13-gramme and 10·5-gramme basis, respectively.

Practically all investigators in India have considered figures mostly well below these levels, so there is little chance that any have been dealing with a purely physiological anæmia.

In the only comparative series quoted (Napier and Bilimoria, *loc. cit.*) there is no evidence of this physiological anæmia, but there is a possible fallacy here, in that, as investigations were being conducted on the anæmia of pregnancy at that time, it is probable that the anæmic pregnant women had been combed out from the pregnant series much more carefully than the non-pregnant anæmics from the other control series; this suggestion is supported by the much higher standard deviation in the non-pregnant series. Napier and Das Gupta (1937*b*) found a distinctly lower level of hæmoglobin amongst pregnant coolies, but noted that when the obviously anæmic women had been excluded the mean was much the same as amongst non-pregnant 'normal' women; they concluded that, whilst evidence of a general lowering of the hæmoglobin level was absent, there was a much higher percentage of gross anæmia amongst the pregnant women.

Where one is dealing with a much higher percentage of pathological anæmia in a population, it is going to be more difficult to recognize the physiological anæmia, but it is a point on which further investigation might be carried out. Meanwhile, it is reasonable to assume that the factors which produce this physiological anæmia in America will also operate in India, though the effect may be disguised.

Bethel (*loc. cit.*) in America found that the size of the cell tended to increase throughout pregnancy reaching its maximum about the 36th week when the hæmoglobin is at its lowest level. He found a mean figure of 86·3 cu.μ for non-pregnant women and 92·0 cu.μ for pregnant women in the 3rd trimester. The only comparable figures in India do not indicate that this occurs here: Napier and Bilimoria (*loc. cit.*) give figures of 77·3 and 72·1 for non-pregnant and pregnant women, respectively. These figures are obtained, it will be seen, in a population in which there is general microcytosis: it is possible that the change in cell volume is an indication of the natural exaggeration during pregnancy of the dominant deficiency; in the Assam group this is an iron deficiency. This is certainly a point on which further work should be done.

Gastric acidity.—This has a double association with anæmia, in that a low gastric acidity has been associated with deficient absorption of iron—though recent work (Moore *et al.*, 1937) does not confirm this—and that free acid is completely absent in true Addisonian pernicious anæmia. Strauss and Castle (1933) pointed out that gastric secretion was often depressed in pregnancy and it has been suggested that this might be a cause of the iron-deficiency anæmia in this state. Bethel (*loc. cit.*) did not find any relationship between gastric secretion and hæmoglobin level in pregnant women.

Wills and Mehta (1930*a*) in a series of 25 pregnant anæmic women found three with achlorhydria, one with hypochlorhydria, and two with hyperchlorhydria: the rest were within normal limits.

There is no evidence that the gastric acidity curve in Indians differs from that of Europeans (Napier and Das Gupta, 1935*d*; Napier, Chaudhuri and Rai

Chaudhuri, 1938; Rao, 1937). Napier and Das Gupta (1937*a*) found, in two series of about 40 cases each, the gastric acidity in anæmic coolies slightly lower than that of normal Indians. Achlorhydria was relatively uncommon; it was found in five cases of the first series (gruel meal without histamin), and in the second series (alcohol test meal) in only one case and in that case at a second examination histamin caused secretion of free acid.

Napier and Majumdar (*loc. cit.*) did fractional gastric analyses on 41 anæmic pregnant women. The majority (26) were isochlorhydric, six were hypochlorhydric and three others achlorhydric (without histamin). Analysed differentially, they showed a lower acid level in the group that responded to liver therapy.

Choudhury and Mangalik (*loc. cit.*) did a gastric analysis in 18 pregnant anæmic cases and found achlorhydria in three and hypochlorhydria in five. Mitra (1937) found achlorhydria in one out of six cases tested, and Chatterjee (Chatterjee and Basu, 1939) 'a low acid curve' in four out of 15 cases. Mudaliar and Rao (*loc. cit.*) provide the only exceptional series and they found four cases of achlorhydria (without histamin) and five of hypochlorhydria in ten cases tested.

There is little suggestion in the data here summarized that there is any connection between gastric acidity and the anæmia of pregnant women in India.

Hæmatological surveys of the anæmias that occur in Indian populations

It is very surprising that, although anæmia is probably second only to fever in the list of most commonly occurring symptoms in any Indian hospital or dispensary, no attempt was made to study anæmia *per se* until a few years ago. This makes the task of reviewing this work comparatively easy. We have not combed the literature for reports on individual cases, or small groups of cases, such as that of Mackie (1929) who read a paper at a meeting of a Medical Society in 1907 on 'malignant anæmia of the tropics'; the paper nearly suffered the fate of the majority of papers read at medical meetings in India and would have been forgotten had its author not found a copy of the typescript and published a résumé of it in 1929. But we found no studies of a larger series of anæmic patients in which any attempt had been made at hæmatological or ætiological classification. This is not altogether surprising in view of the backward state of hæmatology, in India in particular and in the world in general, to which reference has already been made.

Anæmia amongst the general population.—As a preliminary measure to studying the anæmia of pregnancy in the same population, Napier and Das Gupta (1937*a*) investigated the anæmia amongst the general coolie population of a group of tea estates in Assam. Blood examinations before and after treatment were carried out in 100 cases of severe anæmia; these investigations were carried out during two successive cold weathers and the two series are analysed separately. In the first series there were 58 coolies, men and women; as the cell volume estimations were not considered satisfactory, the cases were classified according to the hæmoglobin content of the cells, i.e. the MCH. The vast majority were markedly hypochromic; in 53 cases the MCH was below 28·5$\gamma\gamma$, the mean of the city-dwelling

Indian ' normals ', and 45 were below 22$\gamma\gamma$, the mean of the ' normal ' coolie. Two cases only, with MCH's of 33$\gamma\gamma$, might be considered to be hyperchromic. The cases were arbitrarily divided into two groups, definitely hypochromic, i.e. below 20$\gamma\gamma$, and orthochromic, above 20$\gamma\gamma$.

After analysing the data, clinical and pathological, in these two groups separately, the writers concluded that it was doubtful if they could be considered as two separate hæmatological groups, though the orthochromic group showed a special tendency to hyperbilirubinæmia, with which splenic enlargement was correlated, and achlorhydria, and did not respond so well to treatment with anthelmintics and iron. There were more females in the orthochromic group.

The second series was even more markedly hypochromic; there were only two cases above 22$\gamma\gamma$. The MCV is also reported in this series; two cases would be considered macrocytic judged by some standards, being above 100 cu.μ, but the mean of the series was 69·7 cu.μ and the large majority were below 80 cu.μ. They were therefore not divided, but considered as one group. In this investigation emphasis was laid on treatment and frequent blood examinations were done throughout the course of treatment. Also an attempt was made to gauge the effect of slightly different procedures, including a dietetic experiment.

Eight coolies were kept on a well-balanced (relatively) high-calorie diet for four weeks; there was no improvement in the hæmoglobin level but there was improvement in the clinical picture, including loss of œdema. They were then given large doses of iron (27 grains of ferrous sulphate daily) for three weeks and in four weeks the mean hæmoglobin of the group had risen from 4 to 10 grammes. In the whole series of 41 cases (the 42nd patient absconded), 39 showed definite improvement on iron administration. Though diet *per se* had no effect on the anæmia, the groups in which the diet was most satisfactory showed the best response to the iron treatment. However, in very few cases did the hæmoglobin level improve beyond the level which had been found to be ' normal ' in this coolie population.

It appears to have been shown beyond dispute that the dominant defect, as far as the hæmopoietic system is concerned, in this community is a hypochromic microcytic iron-deficiency anæmia. The cause of this anæmia is not so definite; a hundred per cent of the coolies had a hookworm infection, most of them a heavy infection, but no correlation between the hookworm load and the level of hæmoglobin can be shown. The diet of the coolie is low in iron, but it is uncertain if it is actually deficient; this point will be discussed later. In the absence of any gastric-acid deficiency, which might have led to mal-absorption, it is reasonable to conclude that this anæmia is due to the continual drain, by blood loss through hookworm infection, on the iron reserves in a population on low, if not actually deficient, iron intake. Macdonald (1939) questions this conclusion because of the lack of correlation between the hookworm load and the degree of the anæmia. *But the cent-per-cent hookworm infection cannot be denied*, the methods of estimating hookworm load are open to considerable error, and finally it is only claimed that it is *one* of the factors concerned.

The writers (Napier and Das Gupta, 1937*a*) considered that there was evidence of some other limiting factor which prevents the blood cell returning to its normal size, as in this series the red cells increased to their normal number and the hæmoglobin concentration in each cell, the MCHC, reached the normal level, but, on account of the small size of the cell, it could not contain its full complement of hæmoglobin, so that the MCH and the hæmoglobin per cent only reached the lower, so-called normal, levels of this coolie population; this limiting factor is thus operating not only in the frankly anæmic coolies but in the whole population.

It will be apparent that in this population the anæmia of any particular group, such as the anæmia of pregnant women, must be considered against this background of widespread hypochromic microcytic anæmia and of a general lower level of cell size and hæmoglobin in the whole population. It must also be clear that unless this is done a very wrong impression might be obtained of the nature of such anæmia. No other similar surveys of anæmia amongst the general population in other areas have been reported.

The material for such an analysis with reference to the hospital out-patient population in Calcutta is available, and this analysis will be made. This is certainly a line of work that might well be undertaken in a number of places, but, as in the case of other survey work, when once a lead has been given, it should not be considered as a legitimate charge on funds ear-marked for research. It is a suitable line of work for medical units at colleges or universities.

Anæmia amongst pregnant women.—Balfour (1927*a*) collected hæmatological data in 25 cases of her series. There are no details as to how these cases were selected. She gives the means only of the 25 observations, so that one has no idea of their distribution, but, as she gives 1·4 as the mean colour index, one may assume that the majority were cases of hyperchromic anæmia. McSwiney (*loc. cit.*) makes no attempt to analyse his data, but from his case notes it is apparent that there are both hyperchromic and hypochromic cases in his series.

Wills and Mehta (1930*a*) vitiate their series, as a series, by the exclusion of 16 of their original 66 cases on the grounds that they were suffering from malaria, hookworm infection, syphilis, or definite nephritis; they have classed the remainder as 'idiopathic'. Both the propriety and the possibility of excluding such cases is questioned. The essential difference between the study and the treatment of a case in a sanitarily advanced country on the one hand and in a sanitarily backward country, such as India, on the other is that in the latter one has always to make one's study against a background of widespread infections, such as malaria and hookworm, and of malnutrition, both general and special. So in an attempt to trace the ætiological factor or factors, of a clinical syndrome, one cannot say 'this infection, or this food deficiency, is common to almost the whole population, whereas the syndrome occurs only in a few, therefore we can dismiss it as a possible ætiological factor'. Each infection and each food deficiency must be considered as possible contributing factors whose interactions may produce the syndrome with or without the addition of some metabolic toxæmia, and/or of some physiological stress, such as pregnancy. It would, for example, in the

case of pregnancy anæmia, be absurd to exclude all cases showing ankylostome infections, for in the Assam series this infection occurred in every case almost without exception. Similarly, every woman (in this series) has had malaria at some time during her life, usually both recently and frequently. Further, the possibility of excluding all cases in which the malaria causes the anæmia is doubted, because a single blood examination is not sufficient to exclude even present infections and it is frequent past infections that are as likely to produce anæmia.

Wills and Mehta class their remaining 50 cases as 'idiopathic' and consider them as a single group. From a hæmatological point of view they are not, however, a homogeneous group; the colour indices in the 50 cases vary from 0·7 to 1·8; in 15 it is below 1, in nine from 1 to 1·09 and in 26 cases above this, but in only nine is the colour index 1·40 or more.

Mudaliar and Rao (*loc. cit.*) also treated their cases as a single group. The colour indices suggest that they are not; of 32 cases seven were below 1·0, 14 between 1 and 1·09, and 11 were above this, of which only one was above 1·30. These last workers failed to obtain the same results with Marmite in the treatment of pregnancy anæmia that Wills had reported. This is not surprising, as it is obvious that there are fewer truly hyperchromic cases in their series.

Whilst it is reasonable to assume that all anæmias in pregnancy have not the same ætiology, it is difficult to decide how the cases should be divided up. Wills and Mehta's procedure of excluding all cases in which certain other infections are evident is an attempt to group cases, but is unsatisfactory for the reasons given above. Mitra (1937) however developed this idea; by a more elaborate process of selection he excluded 103 of his 165 cases, leaving 62 idiopathic cases. Nothing in his subsequent analysis of these groups seems to justify this division, except possibly the lower mortality in the 'idiopathic' group. In view of the hæmatological findings this writer reports (e.g. 52 macrocytic cases of which 30 were hypochromic!) his choice of a clinical classification was wise, and, though the result was a negative one, as such, it has some value.

Gupta (*loc. cit.*) classified his cases according to severity, using the red cell count as his criterion—below one million, between one and two millions, etc. There are obvious limitations to such a classification, though it may be usefully adopted for primary grouping when there are large numbers of cases.

The majority of workers in India have adopted some hæmatological classification, either according to the size or the hæmoglobin content of the cell, or both. This method is criticized by Chatterjee and Basu (1938) who claim that during the development of this anæmia the size of the red cell goes through a regular cycle of stages; commencing as normocytic the anæmia becomes microcytic, then if the case deteriorates it passes through a normocytic hyperchromic stage and becomes macrocytic, or conversely if it improves it changes back to normocytic. To explain these strange blood findings they invent a pathological cycle in the bone marrow in which there are successive stages of regeneration, compensation and bone marrow failure. They give examples of cases in which these changes are said to have occurred; the meagre data only show that in the particular cases—errors of technique being

excepted—the blood picture changes from one phase to the next, but proof that a regular cycle occurs in any number of cases is entirely lacking.

The same idea of cyclical changes in the red cells has been entertained by Hare (1939) who considers that there is evidence that the size and the hæmoglobin content of the red cells change according to the stage of the pregnancy. He cites six cases in which the cell size tended to decrease between the 28th and the 36th week, increasing again towards the end of pregnancy or post partum. He points out that the 28th to 36th week is the period of maximum growth of the fœtus. It is interesting that he shows the exact reverse of the course of events that Bethel (*vide supra*) reports in normal pregnancy; Bethel showed that the size of the cell tended to increase throughout the second half of pregnancy reaching its maximum about the 36th week after which it tended to decrease again. Hare's observation, if the changes in these six cases can be accepted as the usual change in the population with which he is dealing, supports the suggestion already made above that a dominant deficiency is exaggerated at this stage of pregnancy by the demands of the fœtus.

Napier and Bilimoria (*loc. cit.*), working amongst the same population in which Napier and Das Gupta (1937*a*) had made a survey of the anæmias of the general population (*vide supra*), divided the cases into groups according to their MCH and hæmoglobin level. Comparing these two groupings, the non-pregnant and the pregnant, it is clear that in the latter series some new factor comes into operation. The hypochromic anæmias are still in the majority, but there is a hyperchromic group, which has been subdivided into two groups according to the severity of anæmia. The less anæmic group, the writers have suggested, might be considered as physiological; this is in keeping with the suggestions made above in which the arbitrary figure of 7·7 grammes, based on Dieckmann and Wegner's (*loc. cit.*) observations, was chosen for coolie populations, and with Bethel's (*loc. cit.*) observations regarding the increase in size of the cell during pregnancy. Amongst the other points that arose in this analysis was the fact that the hypochromic cases tended to come under observation at an earlier stage in the pregnancy than the hyperchromic cases who practically all reported first in the last trimester; the suggestion is that in the latter group the anæmia was precipitated or aggravated by the pregnancy, whereas in the other the pregnancy was an incident in the course of the anæmia. The significantly higher incidence of splenic enlargement in the hyperchromic group has already been referred to, and there was also a 'very significant' preponderance of hyperbilirubinæmia in the hyperchromic groups, as compared with the hypochromic group; in fact, 16 out of 18 of the former group showed a positive indirect van den Bergh reaction, against less than half of the latter. In this series there is a strong association between hookworm infection and the hypochromic group, but not between hookworm infection and the other groups or the controls.

Napier and Majumdar (*loc. cit.*) were working in another district in Assam. Investigations amongst normal coolies had shown that the normal blood picture was of much the same order as had been found in other coolie populations, though the cell size seemed to be larger. (This was to some extent explained by the

inadequacy of the electric current in this district leading to incomplete 'packing' in the cell volume estimations.) No survey of the anæmias of the general population was undertaken here. Hookworm infection was almost a hundred per cent in incidence, but the infections were not so intense as in the other area; on the other hand this was a more malarious district.

The first classification of the cases was, as before, according to the hæmoglobin content of the cells MCH, but, as there was a central hospital in this district, it was possible to follow the results of treatment and later a re-classification of the cases was made according to the effect of treatment, into an iron-responding group and a liver-Marmite-responding group. In this series the initial hæmoglobin level was in no case above 7·7 grammes (*vide supra*). As there were few truly hyperchromic cases in this series, the cases were put into two groups with the dividing line at 22γγ; this divided them into two almost equal groups. Thus, the mean MCH of this series was slightly but distinctly higher than that of the previous series if only the definitely anæmic cases are considered. In the re-classification little change was effected; deaths and failures were excluded and three borderline cases on either side were changed over, otherwise the hypochromic cases became the iron-responding and the orthochromic-hyperchromic the liver-Marmite-responding group. Analysis of the rest of the data according to the two groups showed the high correlation between enlarged spleen and response to liver or Marmite treatment, which has already been referred to, and a very much higher incidence of hyperbilirubinæmia in this same group; thus, there was a positive van den Bergh in 10 per cent of the controls, in 25 per cent of the iron group and in 53 per cent of the liver-Marmite group. There was on the other hand no difference in the hookworm infection between the two groups and a very slightly greater tendency towards a low acidity in the liver-Marmite group.

Sternal punctures were done in a number of cases; the majority showed a normoblastic hyperplasia, and in no case was there the typical megaloblastic hyperplasia that one sees in pernicious anæmia, but in a few cases there were a few megaloblasts and a high percentage of erythroblasts.

A point that was brought out in this investigation was the depressing effect of the fœtus in the later stages of pregnancy on the hæmopoietic response to treatment; this was more noticeable in the liver-Marmite group. When the treatment was given early in pregnancy or after parturition response was usually immediate, but when it was given in the last months of pregnancy there was often a slight reticulocyte response, indicating some specific action, with a very slight upward movement of the hæmoglobin curve or only maintenance of the previous level, until after parturition when there was an immediate sharp rise.

An attempt was made in the first of these inquiries to ascertain the diet of the individual patients, but the method was not found satisfactory, and it was decided that more information could be obtained by dietary surveys amongst the population from which they came. A survey was subsequently carried out in each of the two areas in which these hæmatological investigations have been carried out.

One of these surveys was reported by Wilson and Mitra (1938). The two surveys produced materially the same results:—

The total calories were only a little below the standard usually considered necessary for Indian workers, but were mainly derived from carbohydrates: the total protein intake was low and the animal protein almost a negligible figure. Total fat was also very low, below 12 grammes, and most of it was of vegetable origin. Of the minerals, calcium was exceptionally low, being only about one-sixth of the usual requirements; the figure for iron was low, though adequate if it was all in available form, but there was some doubt on this point. Of the vitamins, the intakes of A and B complex were low, though apparently not actually deficient judged on most standards, but the vitamin-C intake was very low and, as this was mainly derived from vegetables that were cooked, saturation with this vitamin was improbable.

Mitra (1939) re-examined the question of iron intake in the light of later work on the availability of iron and concluded that these people were living on a deficient iron intake. This question of the availability of iron is, however, a controversial one, and whilst it is not possible to accept Mitra's conclusions, it is apparent that people of the poorer classes whose main diet is rice are living on a low available iron intake and that the more this rice is milled the lower will be this intake.

Macdonald (*loc. cit.*), in a separate investigation amongst the same population in which the first of these two inquiries was carried out, found a 'very significantly' lower hæmoglobin level amongst coolies living on milled rice as compared with those taking home-pounded rice.

Choudhury and Mangalik (*loc. cit.*) divided their cases into three groups: macrocytic (MCV over 90 cu.μ and over 29$\gamma\gamma$), microcytic (below these figures), and mixed (MCV above 90 cu.μ and MCH below 29$\gamma\gamma$). There were 27 macrocytic, six microcytic, and eight mixed cases. The indirect van den Bergh was positive in 74·1 per cent of the macrocytic group and in 28·6 per cent of the rest. The MCHC was below 30 per cent in all the hypochromic and mixed cases, and above this in nearly all the macrocytic cases. The high percentage of splenic enlargement in the macrocytic group and its absence in the other has already been referred to.

Conclusion.—The two hæmatological surveys in Assam appear to show that, superimposed on the hypochromic anæmia of the general population, there is hyperchromic anæmia which is associated with the later months of pregnancy. There is also evidence of the association of malaria with the hyperchromic anæmia. As the whole population lives on an ill-balanced diet deficient in a number of elements it seems probable that the hypochromic anæmia can be accounted for by the low intake of available iron and the widespread hookworm infection. Whilst a poor diet and malaria infection are common to the whole community, there is little evidence of hyperchromic anæmia in the general population; it is possible however that a 'slight tendency' to hyperchromic anæmia may be disguised by the marked iron deficiency (this one knows from experience does occur), only to be brought

out as a frank anæmia by the extra demands of pregnancy, or by a toxæmia of pregnancy, which may be a *conditioned* toxæmia, in that it would not occur in a person protected by an adequate diet.

These two surveys have also emphasized the importance of knowing the background against which a disease is being studied. When Balfour first went to Assam to start these investigations she obtained a number of blood films from cases of pregnancy anæmia and sent them to Wills who was working in Bombay. Wills was naturally cautious in arriving at any conclusion from such inadequate material, but she expressed considerable doubts as to whether the same problem existed in Assam as in Bombay, because most of the slides showed a definitely microcytic picture. She was quite right in expressing this opinion on the evidence she had before her, but the data here presented suggests that there is a macrocytic anæmia in Assam associated with pregnancy, and it is probably the same syndrome as is found in Bombay.

The hæmatological picture in the Agra cases (Choudhury and Mangalik, *loc. cit.*) is entirely different; here we have a predominantly macrocytic hyperchromic anæmia, apparently undisguised by any hypochromic tendency in the general population; it is unfortunate that there has been no survey of the anæmia in this population to compare it with. The association of malaria is again suggested by the high incidence of splenic enlargement and hyperbilirubinæmia.

This importance of such surveys in different localities where different ætiological factors prevail must also be apparent. From the inquiry which was recently carried out in Calcutta under Neal Edwards and Napier, similar data will be available when the analysis of the records is complete, but from other places in India, particularly rural districts, practically no information is available.

Summary and conclusions

1. There are good reasons for believing that a high maternal mortality is the rule throughout India. Special investigations have provided figures from 16·6 to the appalling, and probably exceptional, figure of 138 maternal deaths per 1,000 live births, compared with 3·16 per cent in the whole of England and Wales in 1937.

2. Anæmia occupies a high place in the lists of causes of maternal morbidity and mortality in this country; in many places it is second and in some it is first, and, as a contributory cause of death, it is probably even more important than these lists indicate.

3. As in many Western countries anæmia is a negligible cause of maternal mortality, it is obvious that there are special conditions prevalent in India which do not exist, or which exist to a much less extent, in these other countries.

4. The incidence of this anæmia appears to be highest in the second half of the year, even after allowance is made for the greater number of pregnancies at this time. The anæmia usually first attracts medical attention in the third trimester, and the incidence is apparently slightly higher amongst primiparæ.

5. Œdema occurs in nearly all cases of pregnancy anæmia, and fever in more than half; albumin is found in the urine in 25 to 50 per cent of cases, and diarrhœa occurs in a similar percentage. A relatively high incidence of splenic enlargement is reported by most workers, particularly amongst macrocytic cases, and some have noted a higher incidence of Wassermann-positive cases than occurs amongst the general population.

6. It has been shown that the blood picture of the normal healthy city-dwelling Indian living on a good mixed diet conforms closely to the standards that are based on observations made amongst Europeans in Europe and America, but, on the other hand, that in rural populations and in Indians of the lower economic strata, the hæmoglobin level of the whole population may be much lower than the above-mentioned standards and there may be other evidence in the blood picture of dys-hæmopoiesis; whilst these observations apply also to the so-called normal individuals, there are in the latter populations a large number who show definite clinical signs of anæmia.

7. With rare exceptions, the ætiological factors, in so far as they have been identified, that produce clinical anæmia, whilst they exert their greatest effect in the case of the frankly anæmic individual, also affect the individuals of the general population to a greater or lesser extent, and it seems reasonable to assume that these are the factors mainly responsible for producing the state of dys-hæmopoiesis widespread in these particular populations.

8. It has been shown that amongst the general populations in places where the anæmia has been studied there are three types of anæmia that are very common. These are:—

(*a*) A microcytic hypochromic iron-deficiency anæmia due to living on a diet actually deficient in available iron, or to continuous loss of blood, usually from hookworm infection, in people living on a diet low in available iron.

(*b*) A macrocytic hyperchromic anæmia due to a deficiency in the diet of some substance that is present in autolysed yeast and in crude liver extracts, which anæmia is distinguishable from pernicious anæmia by the presence of free acid in the gastric secretions, the absence of neurological symptoms, a negative van den Bergh (indirect) test, and the absence of typical response to purified liver extracts (Anahæmin).

(*c*) A second type of macrocytic hyperchromic anæmia, distinguishable from the other type by evidence of increased hæmolysis—namely, hyperbilirubinæmia (a positive van den Bergh reaction) and a high reticulocyte percentage—and usually splenomegaly, which is probably associated with chronic malaria *and* some degree of dietary deficiency.

9. It is generally recognized that there is normally in the later months of pregnancy an anæmia, which may be a purely physiological anæmia, or due to a relative dietary (? protein) deficiency and which is common in many communities in other countries; with this anæmia is associated an increase in the mean corpuscular volume of the red cells.

10. The incidence of anæmia in pregnancy in India appears to be correlated with that of the anæmia in the general population, but in pregnancy the incidence is higher and the condition assumes greater importance.

11. In pregnancy the anæmia shows a more marked macrocytic tendency than the anæmia in the general population, though in some localities this is almost completely disguised by the associated iron deficiency (or other microcytic anæmia-producing factor).

12. Anæmia in pregnancy is amenable to suitable treatment in the early months of pregnancy, and in some cases in the later months but in the majority of these it is not possible to stop or slow down the deterioration of the blood picture until after parturition, when an immediate and rapid improvement commences in practically all except those cases complicated by sepsis. This sudden and rapid improvement is comparable to the sharp rise in hæmoglobin that occurs in certain cases of malaria when the infection is controlled by efficient treatment; this suggests that the inhibitory factor is of the nature of a toxæmia associated with the actual presence of the fœtus.

13. The evidence so far collected appears to point to the fact that anæmia in pregnancy is due to a combination of the ætiological factors that produce anæmia in the general population with some factor associated with pregnancy itself and the presence of the fœtus.

Some of the common ætiological factors are known, probably others are still to be discovered: of those that are known, the most important are hookworm infection and a low available iron intake, as the cause of microcytic anæmia, and specific and general dietary deficiency (e.g. absence of the specific factor that occurs in autolysed yeast and of biologically-valuable protein), alone or in combination with chronic malarial infection, with its associated splenomegaly, reticulo-endothelial proliferation, and increased blood destruction, as the causes of macrocytic anæmia.

These factors, together with the specific pregnancy factor, vary in their influence in different populations that have been investigated and in different individuals, and produce a variety of blood pictures microcytic and hypochromic, macrocytic and hyperchromic, and, where two opposing influences are acting more-or-less equally in the same individual, normocytic and hypochromic, macrocytic or normocytic and orthochromic, or rarely some other combination.

The effect of pregnancy itself, it has been suggested, is solely in the extra demands of that inexorable parasite, the fœtus, of some essential food substance; this suggestion is supported by the fact that in the sub-clinical anæmia of pregnancy the peak of the blood dyscrasia is correlated with the period of maximum growth of the fœtus. There is, however, in the writer's opinion, evidence, in addition, of some toxæmia associated with the actual presence of the fœtus, which inhibits hæmopoietic response in severe forms of anæmia when in the later months of pregnancy the deficient substances are administered even in full doses, although, after the uterus has been emptied, there is an immediate maximal response; the toxæmia is probably a 'conditioned toxæmia' which only operates when there is an associated specific food deficiency, either absolute or relative.

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PART II

HÆMATOLOGICAL TECHNIQUE

1

The collection of the blood sample

THE first essential is that one constant practice should be decided upon and followed. There is a difference between capillary. which for all practical purposes is arterial, and venous blood, but the difference is only of practical importance if two samples from the same individuals at different times are to be compared, or when normal standards are being worked out.

As there are very distinct advantages in the use of venous rather than capillary blood, the former is preferred.

The advantages are that all the examinations, e.g. the estimation of hæmoglobin, the determination of cell volume, the total red and white cell counts, the reticulocyte count, the differential white cell count, the measuring of the red cell diameters, the icterus index, the van den Bergh reaction, the fragility test, the erythrocyte sedimentation test, etc., can all be done from a single sample of blood obtained by a single puncture. Further, the tests can be repeated by the same or by different workers from the same sample of blood.

For some of the tests, the van den Bergh test for example, blood will have to be taken from the vein, so that sufficient might as well be taken at one time for all the tests and the patient saved a number of additional pricks. In weak and anæmic individuals, it is sometimes difficult to draw enough blood from a finger or ear lobe without squeezing the part, and thereby diluting the blood with the tissue fluid.

The following rules should be observed when using venous blood :—

(*a*) The syringe should be air-tight and perfectly dry. The syringe is conveniently dried by first washing it thoroughly in clean water, removing the water with alcohol, removing the alcohol with ether, and finally drawing air through it. If there is even a trace of water or alcohol, there will be hæmolysis of the blood, and this will make it unsuitable for examination.

(*b*) The tourniquet should not be kept tied for more than 2 minutes when drawing the blood from the vein, as the red cell count begins to increase after 3 minutes' stasis.

(*c*) The needle of the syringe should be removed before the blood is put into the flask, as hæmolysis may result if the blood is forced through the needle.

(*d*) The flask should always be kept closed with a rubber cork; cotton-wool should never be used. The flask should be shaken at once vigorously, to ensure complete solution of the oxalate powder.

(*e*) The flask must be kept corked when not in use, and in a cool receptacle during the summer.

(*f*) The blood should always be mixed thoroughly for at least 3 minutes, preferably in a shaking machine, before any sample is withdrawn from it. The blood should always be taken directly from the flask and not from blood poured out on to a slide or watch-glass.

Time for collection of blood.—When repeated examination is required, blood should always be collected at the same hour under basal conditions in the morning. Even for a single examination it is preferable to collect the blood in the morning under basal conditions; this precaution will minimize the effect of the fluctuations in the total and differential count due to muscular and other physiological activities.

Procedure.—The blood is drawn from one of the prominent superficial veins at the bend of the elbow in a perfectly dry air-tight syringe.

First of all, without detaching the needle, small drops of blood are put on clean glass slides, and smears for the differential leucocyte count and red cell diameter measurements should be made*; then the needle is detached and a measured amount of blood is transferred to a small 25 c.cm. Erlenmeyer flask containing the requisite amount of anti-coagulant for the quantity of blood to be taken.

Anti-coagulant.—Two milligrammes of dry oxalate powder is required for each cubic centimetre of blood. A mixture of potassium and ammonium oxalate powder in the proportion of 2 to 3 has been found to be ideal, as, in the above proportion, the shrinkage caused by potassium oxalate is counteracted by ammonium oxalate (Wintrobe and Landsberg, 1935). To measure the requisite amount of the oxalate powders make a 1 per cent solution of the two oxalate powders, ammonium and potassium; accurately measure with a pipette 0·4 c.cm. of the potassium salt and 0·6 c.cm. of the ammonium salt, and put them into the flask; evaporate in a dry oven, after which the oxalate will be found at the bottom of the flask in a powdered state; this is the amount required for 5 c.cm. of blood; for 3 c.cm. the proportions will be 0·24 c.cm. potassium and 0·36 c.cm. ammonium salt. The flasks are now kept corked and are ready for use.

It is a good practice to have two sets of flasks prepared containing enough anti-coagulant for 5 c.cm. and 3 c.cm., respectively.

If potassium oxalate alone is used, 2 milligrammes are required for each cubic centimetre of blood, that is, 10 milligrammes or 1 c.cm. of 1 per cent solution for each 5 c.cm. of blood, and in this case Wintrobe's correcting factor × 1·09 must be applied to compensate for shrinkage.

* As stated above this can be done from the oxalated sample, but it is better to make separate smears.

Time limits.—Osgood and others (1931) give the following time limits for different examinations:—

Hæmoglobin estimation, red cell count, white cell count, and reticulocyte count—24 hours.

Icterus index and van den Bergh test—2 hours.

Cell volume, fragility test, and sedimentation rate—3 hours.

Making smears for differential count—1 hour.

2

Estimation of haemoglobin

To estimate the amount of hæmoglobin in any sample of blood advantage is taken of certain properties of hæmoglobin. Of these the most important is its oxygen-carrying capacity. A fixed amount of hæmoglobin will always combine with the same amount of oxygen, actually 1 gramme of hæmoglobin with 1·34 c.cm. of oxygen: the amount of oxygen that will combine with a sample of blood can be measured accurately and the hæmoglobin content of the sample thus estimated.

Another property is its iron content. Hæmoglobin has a known molecular formula in which there is one atom of iron, so that by estimating the amount of iron in the red cells from a given volume of blood it is possible to calculate the amount of hæmoglobin in the sample.

Hæmoglobin is a protein with a known refractometric index. By measuring this index it is possible to calculate the amount of hæmoglobin in any sample.

Yet another property of hæmoglobin is its colour and this can be measured in the various ways indicated below.

Whilst the property of hæmoglobin easiest to measure is its colour, the most fundamentally important property is its oxygen-carrying capacity. There is evidence that these two properties are very closely correlated, so that if an instrument which measures the colour is first calibrated by the oxygen-carrying method a very close approximation of the hæmoglobin content of a sample can be obtained by the former method.

Most clinical hæmoglobinometers that are now sold are thus calibrated.

Clinical methods of estimating hæmoglobin.—Of the many methods of estimating hæmoglobin that are in use, none of the simpler methods is above criticism, while the more accurate procedures, e.g. van Slyke's oxygen-carrying-capacity method, are hardly suitable for general clinical practice. Most of the clinical procedures in common use are methods of measuring the colour of the hæmoglobin and are based on one or other of the following principles:—

I. Direct comparison of the colour of undiluted blood against a graduated colour standard.

(*a*) On special blotting paper (Tallqvist).

(*b*) Between glass plates (Dare).

II. Comparison of blood diluted to a fixed percentage against a graduated colour standard.

(*a*) Diluted with sodium carbonate solution and compared with a coloured glass wedge (Fleischl-Miescher).

(*b*) Diluted with deci-normal hydrochloric acid and compared with coloured glass wedge (Hellige-Neoplan).

III. Comparison of blood diluted to a varying degree with a fixed colour standard.

(*a*) Diluted with water and compared with a permanent picrocarmine standard (Gowers).

(*b*) Brought into contact with carbon monoxide, diluted with water, and compared with a permanent colour standard (Haldane).

(*c*) Diluted with deci-normal hydrochloric acid and compared with a permanent standard of acid hæmatin (Sahli), or a coloured glass block (Hellige).

IV. Comparison of blood diluted with deci-normal hydrochloric acid to a fixed percentage against a fixed colour standard in a colorimeter of the Duboscq type.

(*a*) Water placed in one chamber of the colorimeter and a standard coloured disc interposed (Newcomer).

(*b*) Comparison with an acid hæmatin solution of known strength.

Other methods which require more elaborate instruments depend on the intensity rather than the colour of the light transmitted through hæmoglobin solutions; this is measured by means of a photo-electric cell.

In the writers' laboratory the new Hellige 'normal hæmometer' is used for all clinical purposes. The great advantage in the Hellige instrument is that it is possible to match the brown-coloured acidulated blood solution with the colour standard exactly, and, as the standard is made of coloured glass, it is unlikely to undergo any colour change; one coloured prism which has been in use in our laboratory for more than four years has not undergone any colour change during this period. Error, excluding those of carelessness in technique, using dirty pipettes, etc., may arise through variations in the diluting pipettes and in the calibres of the graduated tubes. The method suffers the disadvantage that with each dilution only one reading can be made—a second observer can check the reading, but not make an independent one—and that a time interval of 15 to 20 minutes must be allowed before reading the result.

Methods of expressing the results.—The amount of hæmoglobin in an individual's blood may be expressed—

(*i*) As the number of grammes per 100 c.cm. of blood, or (*ii*) as the percentage of the amount present in the blood of the 'normal' individual.

The disadvantage in the latter method is that there is no uniformity of opinion as to what is a 'normal' individual. Figures for the 'normal' given by different writers vary from 13·8 to 17·3 grammes, and in our personal experience we have

found amongst groups of so-called healthy coolies in Assam a figure lower than the former, and amongst healthy Europeans in India one higher than the latter. If the expression '100 per cent hæmoglobin' has any meaning at all, it must imply that the particular sample is what one would expect from that individual if he were in perfect health. This would mean that a different standard would have to be adopted for each class of individual, a procedure which would lead to endless confusion. It is therefore better to abandon the second method, namely, that of giving the hæmoglobin in terms of a percentage of an unknown and arbitrary standard, and to adopt the first and express results in terms of grammes of dry hæmoglobin per 100 c.cm. of blood.

Instrument makers, who have hitherto made no attempt to adopt a universal standard, and hæmatologists, who have not encouraged them to do so, are both responsible for the present unsatisfactory state of affairs.

The confusion that exists cannot be better exemplified than by the table (I) below which shows that not only are there differences in the standard between instruments of various makes, but instruments of the same name made at different times vary from one another, and finally various authorities give different figures for apparently the same instruments.

TABLE I

Amounts of hæmoglobin per 100 *c.cm. of blood which are shown as* 100 *per cent by different instruments*

Authority	Tallqvist	Sahli	Dare	Newcomer	Haldane	Fleischl-Miescher
Pepper and Farley, 1933.	13·8	17·3	13·77 (old) 16·9 (new)	..	13·8	15·8
Nicholson, 1934 ..	15·8	17·2 (old) 14·5 (new)	13·8	16·9	..	..
Whitby and Britton, 1939.	13·8	17·3 (old)	15·9	..	13·8	..
Kracke and Garver, 1937.	15·8	17·3 (old) 13·8 (new)	13·77	16·9	..	..
Levinson and MacFate, 1937.	15·8	17·3	16·0	15·92	..	..
Ordway, Gorham and Issacs, 1937.	15·8	13·8* to 17·2	13·7 to 16·0	16·9	13·8	15·8
Beck, 1938 ..	13·8	13·8* to 17·3	13·7* to 16·9	16·9	..	..

* Different instruments are supplied with different standards.

However, many instruments that are now sold give the readings in terms of grammes of hæmoglobin per 100 c.cm. of blood; others give readings in duplicate so that it is easy to see what corresponds to 100 per cent, or, if they use the older method, they state what their 100 per cent corresponds to in terms of dry hæmoglobin.

The excuse given for adhering to the older method (*ii*) is that most practitioners are familiar with this method of expressing the hæmoglobin. It may be true that they are familiar with the expression, say, 'hæmoglobin 80 per cent', but they don't know what it means, for it may mean anything between 11 and 13·8 grammes of hæmoglobin per 100 c.cm. of blood, a discrepancy which is far from negligible. They must therefore learn something new, so why should they not learn a new method which has a definite meaning accepted by all hæmatologists. Most American medical books are now using the 'new nomenclature' and in Great Britain even the more conservative clinicians will follow the lead already given by British hæmatologists, in course of time.

A table (II) showing the 'normal' hæmoglobin levels in different populations in India with a few samples from other countries for comparison, is given.

Hellige normal hæmometer

The instrument consists of the following parts:—

(*i*) A mixing tube with graduations from 10 to 170 (*see* figure 1, A).

(*ii*) A pipette with a mark at 20 c.mm. capacity (B).

(*iii*) A solid glass rod for stirring the mixture in the tube to ensure thorough mixing.

(*iv*) Coloured prism, or prisms, against which the blood has to be matched (C).

(*v*) The housing of the hæmometer (D) which is made of steel with a large base to ensure safe standing. Inside the housing are the coloured prisms in the front and an opaque glass plate at the back. Through a hole in the housing the mixing tube is introduced and lies at the same distance from the eye as the prism.

Technique.—Fill the graduated measuring tube up to mark 10 (using a large pipette) with N/10 hydrochloric acid.

After shaking the blood in the flask for 3 minutes, with the pipette draw up the blood to the 20 c.mm. mark exactly, wipe away any blood adhering to the outside of the pipette, and transfer the blood into the measuring tube. If the blood goes a little beyond the mark, it is brought back to the mark by touching one's finger with the tip of the pipette a few times; cotton-wool or blotting paper should never be used.

By repeated filling and emptying, the pipette should be completely freed from all vestiges of blood, and the blood should be intimately mixed with the hydrochloric acid; the red hæmoglobin now turns to acid hæmatin (brown).

TABLE II

'Normal' hæmoglobin levels of different populations

Sex	Age	Locality	Economic status	Hæmoglobin in grammes per 100 c.cm.	Standard deviation	Number on which based	Authority
Men ..	19–30	Bombay	Students	15·37	± 0·96	121	Sokhey *et al.*, 1937.
	25–45	Calcutta	Mixed, servants.	14·77	± 1·38	50	Napier and Das Gupta, 1935*a*.
	25–45	„	Clerks and doctors.	15·70	± 0·91	30	„ „ „ 1936.
	Adults	Assam	Coolies	12·63	± 1·41	20	„ „ „ 1936.
	„	Cachar	„	12·60	± 1·83	25	Napier and Majumdar, 1938.
	„	Assam	„	11·83	± 1·07	24	Napier and Das Gupta, 1935*b*.
	„	Shivrajpur	„	13·74	± 1·70	47	Sen (Napier, 1939).
	„		„	12·95	± 1·72	49	„ („ „).
	„	U. S. A.	..	16·00	..	..	Castle and Minot, 1936.
	„	Britain	..	15·60	..	..	Whitby and Britton, 1939.
	„	„	..	14·50	..	..	Price-Jones, 1931.
Women ..	18–30	Bombay	..	12·99	± 1·10	101	Sokhey *et al.*, 1938.
	14–38	Calcutta	Middle class	12·63	± 1·01	128	Napier, Edwards and Das Gupta, 1941.
	17–22	Madras	Students	13·73	± 0·93	62	Sankaran and Rajagopal, 1938.
	17–30	Delhi	Middle class	13·11	± 0·81	100	Benjamin, 1939.
	Child bearing	Coonoor (6,000 feet).	..	15·81	± 2·54	100	Radhakrishna Rao, 1938.
		Cachar	Coolies.	10·40	± 1·74	25	Napier and Majumdar, 1938.
		Assam	„	10·80	± 2·30	20	Napier and Bilimoria, 1937.
		Britain	..	13·60	..	..	Price-Jones, 1931.
		„	..	13·70	..	..	Whitby and Britton, 1939.
	18–22	Michigan	Students	13·76	..	50	Bethel, 1936.
Pregnant women.	..	Assam	Coolies	10·70	± 1·60	40	Napier and Bilimoria, 1937.
„	..	„	„	9·22	..	228	Napier and Das Gupta, 1937*b*.
„	..	„	(Obvious anæmics exeluded).	9·99	± 1·72	192	„ „ „ 1937*b*.
„	..	Michigan	..	11·85	..	28	Bethel, 1936.
„	..	Coonoor (6,000 feet).	Ante-natal clinic (mixed).	15·52	± 2·52	100	Radhakrishna Rao, 1938.

Wait for 15 to 20 minutes; the mixture which is now brown should be perfectly clear.

Now add water slowly drop by drop with a pipette, mixing constantly with the solid glass rod, until the mixture matches exactly the colour of the standard prism C in the housing. During the process the solid rod should not be placed on the table, as each time it is allowed to touch anything the small amount of mixture adhering to the rod will be lost, and this source of error will be multiplied.

To match the solution and the standard, the hæmometer should be held up to a good source of indirect natural light, direct sunlight and artificial light being avoided. The level in the mixing tube opposite the lowest point of the meniscus of the diluted acid hæmatin is read; this gives the percentage of hæmoglobin. The end point is generally very sharp and there is seldom any difficulty in matching the solution and standards exactly, except with leukæmic and jaundiced blood. When a point is reached at which the solution appears to match the standards, take a reading; then add a drop of water, mix the solution, hold up to the light and take a second reading. If at the second reading the solution is definitely too light the first reading should be taken. On the other hand if the second reading still appears to match the standards, but, after yet another drop has been added, the solution is definitely lighter than the standards, the result should be the mean of the first two readings.

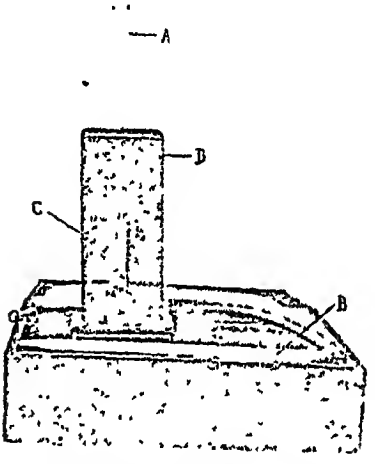


FIG. 1.—Hellige normal hæmometer.

The present writers, Napier and Das Gupta (1935), calculated that 100 per cent with the Hellige normal hæmometer represented 13·67 grammes by the refractometer method, but later by means of the van Slyke oxygen-carrying method a slightly higher figure was obtained and they decided to consider 100 per cent as 13·75 grammes.

Other instruments of this pattern (Hellige normal hæmometer) that they have used have not differed appreciably from this standard, but it is advisable to have the colour standard with the pipettes and mixing tubes to be used, tested in some laboratory where the van Slyke oxygen-carrying method is also employed.

The reading in terms of percentage from the tube must be converted into grammes; this can be done conveniently by using the conversion table of which the skeleton is given (Table III). It is recommended that the full table be

prepared and kept handy, though any figures between 2 and 109 not given can be calculated by summing two figures.

TABLE III

Skeleton table of hæmoglobin values of the Hellige normal hæmometer converted into grammes of hæmoglobin per 100 *c.cm. of blood*

Per cent Hellige	Grammes per 100 c.cm.	Per cent Hellige	Grammes per 100 c.cm.
1	0·1375	10	1·375
2	0·2750	20	2·750
3	0·4125	30	4·125
4	0·5500	40	5·500
5	0·6875	50	6·875
6	0·8250	60	8·250
7	0·9625	70	9·625
8	1·1000	80	11·000
9	1·2375	90	12·375

100 per cent Hellige = 13·75 grammes per 100 c.cm.

3

Enumeration of erythrocytes in the peripheral blood

Erythrocyte and leucocyte counts are expressed as the number of cells per cubic millimetre (c.mm.) of blood.

Principle.—In enumerating the red or white cells, oxalated venous blood from the flask (or capillary blood from the finger or ear lobe) is taken into a red or white cell pipette up to a certain mark, and the pipette is filled with the diluting fluid; it is then shaken, and a drop of the mixture is put into a special counting chamber, where the cells within some specified ruled area are counted, and finally the total number of cells per c.mm. is calculated.

The dexterity necessary to carry out the various manipulations in the different stages of making a blood count can only be acquired by practice. Further, close attention must be paid to every detail of the technique, if accurate results are to be obtained.

Apparatus required

(*i*) Microscope.
(*ii*) Hæmocytometer with counting chamber, preferably with Neubauer ruling.
(*iii*) Red and white cell pipettes.
(*iv*) Hæmocytometer coverslip.
(*v*) Diluting fluids.
(*vi*) Watch-glass.

The ideal would be to use certified counting chambers and pipettes, but, where this is not possible on account of their high cost, only those which are manufactured by reliable firms (viz. Carl Zeiss, Bausch and Lomb, etc.) should be used and these should at least be checked against certified instruments.

Counting chambers. –There are various types of counting chamber in use. The new types of chamber have two main advantages over the old Thoma chamber:—

(1) The coverslip can be placed in position upon its base before the blood is introduced and this may be done slowly and with the care that is essential to ensure the appearance and persistence of Newton's colour bands at the surfaces in contact.

(2) The uneven distribution of the corpuscles on the counting surface, which is liable to arise in the original chamber devised by Thoma and is due to the rapidity with which the corpuscles, especially the red ones, tend to settle in the diluent, is obviated; the new chambers are so devised that the diluted blood rapidly enters the counting space by capillary attraction.

In the new types of chamber, e.g. Neubauer, Bürker, etc., the counting space is oblong and divided into two compartments by a transverse groove, both of which contain a ruled area for counting the red as well as the white corpuscles. These two counting areas afford a means of making duplicate counts with a single application of the coverslip.

In our laboratory we have used chambers with Thoma, Bürker and Neubauer rulings, but for over two years we have been using only chambers with the Neubauer ruling which is simpler to use than the Bürker; with a single application of the coverslip, a larger area for the leucocyte count, 4 square millimetres on each side of the chamber, as opposed to 1 square millimetre in the Thoma type, is obtained.

Counting chamber with improved Neubauer ruling

The different parts of the counting chamber are shown in figures 2 and 3.

The middle platform A-A1 is exactly 0·1 mm. lower than the two side platforms. When the coverslip is placed upon the platform, there is a space exactly 0·1 millimetre deep between it and the ruled area on the platform.

Upon the ruled area, there are nine large squares, 1 square millimetre each, separated by double lines.

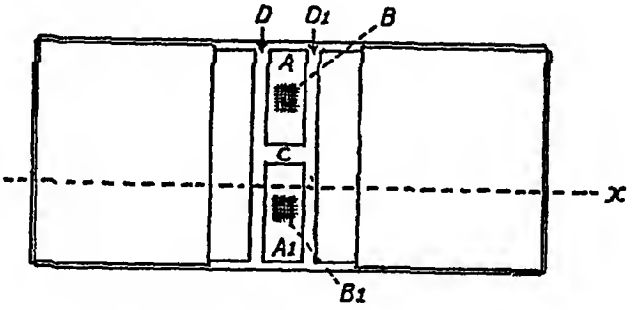


FIG. 2.—Improved counting chamber.

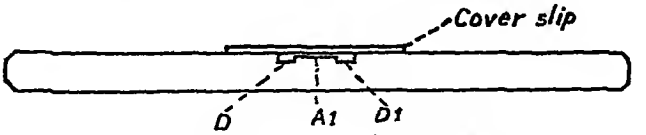


FIG. 3.—Section at 'x' of figure 2.

The four large corner squares A, B, C and D, are the areas in which the leucocyte count is made (figure 4). Each of these 4 square millimetres is subdivided into 16 squares to facilitate counting.

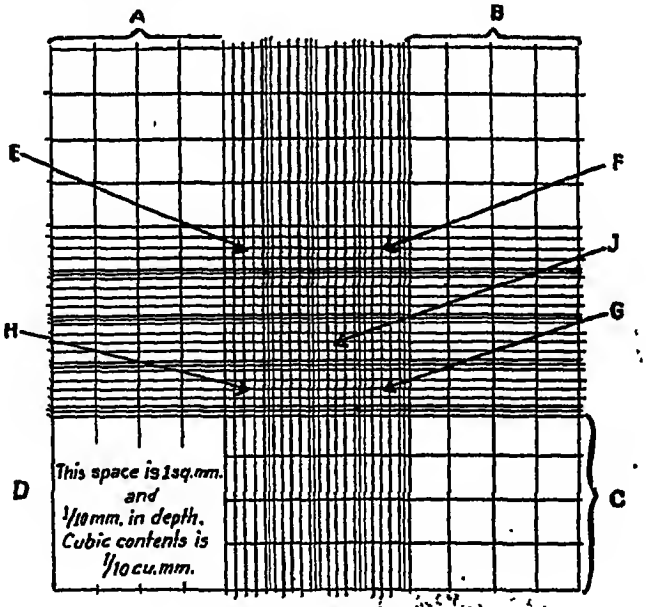
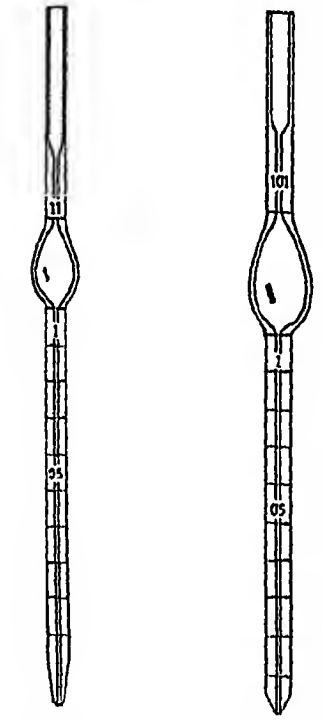


FIG. 4.—Neubauer ruling.

The square millimetre in the centre of the ruled area is used for the red cell count. It is subdivided into 400 smaller squares, each of which is therefore $\frac{1}{400}$ of a square millimetre.

To facilitate counting, these 400 small squares are cut up into 16 groups of 16 small squares each, by extra lines which are drawn through every fifth square. Red cells in the five small groups of squares, one from each corner, E, F, G and H and one from the centre, J, that is to say, the contents of 80 small squares in all, are counted.



Leucocyte. Red cell.

FIG. 5.—Counting pipettes.

The coverslip.—Only the coverslip which is specially designed for blood-counting chambers must be used. If any other coverslip is used there may not be uniform depth in the counting chamber and the count will be inaccurate.

The blood-counting pipettes (Thoma type)

The capillary portion of both the red and white cell pipettes (figure 5) is divided into ten equal parts from the tip to the bottom of the bulb, the fifth and the tenth marks being denoted by figures 0·5 and 1, respectively. There is another mark just above the bulb where the figure 101 is given on the red cell pipette and the figure 11 on the white cell pipette.

To facilitate mixing, there is a bead in the bulb, which is sometimes coloured red in the red cell pipette to distinguish it from the white cell pipette.

Diluting fluids.—For the red cell count we have found the following solution the most satisfactory:—

Sodium sulphate	..	..	..	12·5 grammes
Glacial acetic acid	..	..	..	33·3 c.cm.
Distilled water	..	..	..	200 „

There is neither clumping nor hæmolysis with this fluid. Other solutions recommended are Hayem's fluid,

Mercuric chloride	..	..	..	0·5 gramme
Sodium chloride	..	..	..	1·0 „
Sodium sulphate	..	..	..	5·0 grammes
Distilled water	..	..	..	200 c.cm.

and simple physiological salt solution. We have not found either of these satisfactory, as clumping may occur with the former and hæmolysis with the latter solution.

For the white cell count we have found the following solution the best:—

Glacial acetic acid	..	..	..	2 c.cm.
Mercuric chloride	..	..	..	0·1 gramme
Aniline gentian violet	..	..	..	one drop
Water	..	..	..	to 100 c.cm.

The mercuric chloride will prevent growth of moulds when the solution is kept for a long time, and the gentian violet will give a slight tinge to the leucocytes and also make it easy to distinguish this solution from the red cell diluting fluid.

When the diluting fluids are used, a small quantity should be put into a watch-glass or other suitable receptacle into which the pipettes charged with blood should be plunged. In no case should the pipettes be put into the original phials containing the diluting fluids, as there is every possibility that in course of time cells will find their way into this fluid and accumulate at the bottom of the phial; these may be taken up subsequently with the diluting fluid and vitiate the count.

Enumeration of erythrocytes

(*i*) *Filling the pipette.*—Before using a pipette see that it is absolutely dry and clean and that the point is intact (the tips are easily damaged). With a dry red cell pipette suck blood up to the mark 0·5 (or 1·0, in the case of anæmic patients) by holding the pipette almost horizontally and at right angles to the line of vision, so that the exact height of the column of blood can be seen easily. The blood should not go much beyond the mark, but, if it does go a little beyond, it is brought back to the mark by applying the tip of the pipette to the tip of the finger a few times; cotton-wool or blotting paper should never be used as they will draw out the serum only and the cells in the pipette will become more concentrated. The blood adhering to the outside of the pipette is wiped off, and the pipette is plunged into the red cell diluting fluid in a watch-glass. The diluting fluid is drawn up exactly to the mark 101, the pipette being held nearly vertical and gently rotated between the thumb and the forefinger. The rubber tube is taken off and a tightly fitting flat rubber band is put round the pipette closing both the ends to prevent any leakage. It is now laid flat on the table, or in the box.

When the pipette is filled, it will be seen that the entire capillary end of the pipette from the tip to mark 1 is occupied by diluting fluid. Hence, the true dilution of the cells is not 0·5 (or 1) in 101, but 0·5 (or 1) in 100, i.e. 1 in 200 (or 1 in 100). Thus, by taking blood up to the different marks from 0·1 to 1 in the pipette, dilutions ranging from 1 in 1,000 to 1 in 100 can be made.

Should there be any air bubbles at any stage during the filling of the tube with the blood or the diluting fluid, the whole preparation must be rejected and another pipette filled up, for it is never possible to get all the air out without loss of some fluid.

(*ii*) *Shaking the pipette.*—On every occasion, prior to charging the counting chamber with the diluted blood, the pipette should be shaken, preferably in a

shaking machine or by rotating the tube, held horizontally, between the palms of the hands, first with the right hand on top and then the left, for about 2 minutes. The pipette should never be shaken in a horizontal direction, i.e. in the direction of its long axis, as this tends to throw the cells into the capillary tube.

(*iii*) *Charging the counting chamber.*—The counting chamber and the coverslip must be perfectly clean and free from dust or threads of cotton. Put the coverslip on the counting chamber so that it covers the ruled areas B and B^1 (figure 2), and with the finger nail tap the coverslip to ensure good contact. Discard the first few drops of the diluted blood by blowing into the pipette. Next, holding the pipette in an inclined position of about 45 degrees, apply a small drop of diluted blood to that part of the platform A-A^1 of the counting chamber which just projects from the coverslip; the drop will instantly pass under the coverslip by capillary attraction. The drop of blood should not be too large, or it will overflow the chamber and pass into the overflow trenches D-D^1, and may even pass to the transverse trench C. Neither should the drop be too small as in that case it will not completely fill the counting surface. With a little practice a drop of the requisite volume can be put under the coverslip; any little excess on the platform should be removed by sucking with the pipette and never with blotting paper.

Small air bubbles are liable to be introduced into the counting chamber together with the diluted blood :—

(*i*) if the counting surface and the coverslip are not previously cleaned with the requisite care, or

(*ii*) if the point of the pipette is chipped.

If there be any overflow into the trenches, or if there are any air bubbles remaining, the chamber must be cleaned and charged again.

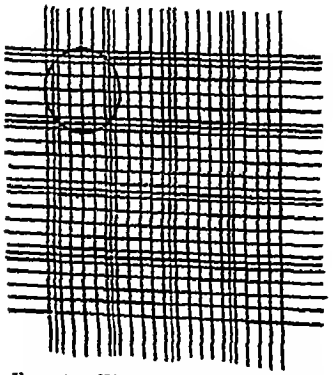


Fig. 6.—Thoma ruling as seen with two-third lens. The black circle shows the field seen with the one-sixth lens.

Place the counting chamber on the microscope stage or on any horizontal surface for about 2 to 3 minutes to allow the cells to settle properly.

Focusing the ruled area

Great care is necessary in locating the ruled area without injuring the objective or disturbing the distribution of cells. This is most conveniently done by locating the ruled area first with the low power (two-thirds objective and 10 × eyepiece)—lowering the condenser or reducing the light by means of the iris diaphragm will show up the rulings distinctly (figure 6); the higher power (one-sixth objective) may then be substituted by simply rotating the nose piece of the microscope and adjusting the condenser and diaphragm.

Counting the cells

Focus the ruled area with the low power and also find out if the cells are evenly distributed—if not, another preparation should be made.

The cells in the squares can now be counted with the high power, one-sixth objective. The area from which the cells are to be counted will vary with the different types of ruling, but the same rule for cells on boundary lines should hold good in all cases.

Rules for cells on boundary lines.—All the corpuscles touching the upper line and the left hand line of a square are considered to be inside the square, while those touching the lower line and the right hand line of a square are considered to be outside the square (figure 7).

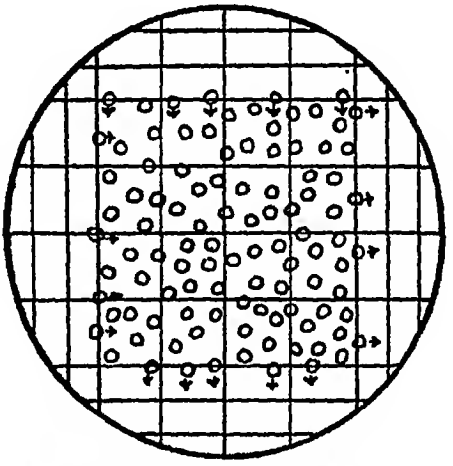


FIG. 7.—One field with one-sixth lens showing which cells should be counted and which neglected.

The cells contained on the five groups of small squares E, F, G, H and J in the middle of the ruled area of the Neubauer ruling (figure 4) are counted.

In all cases the cells on both sides of the chamber should be counted and the mean of the two taken. Should there be a difference of more than 5 per cent in the two counts, the chamber should be recharged and the count done again.

Calculations for erythrocyte count

Each small square $= \frac{1}{400}$ square millimetre.

Therefore, the cubic dimension of each $= \frac{1}{400} \times 0{\cdot}1$ (space between the coverslip and the ruled area) $= \frac{1}{4000}$ cubic millimetre.

And each group of 16 small squares $= \frac{16}{4000} = \frac{1}{250}$ cubic millimetre.

Therefore, cells from five groups of 16 squares each $= \frac{5}{250} = \frac{1}{50}$ cubic millimetre of diluted blood.

Thus, the total number of cells counted from five groups of small squares multiplied by 50 will give the number of cells in 1 c.mm. of diluted blood.

This, when multiplied by the diluting factor 200 (or 100) will give the number of cells in one cubic millimetre of blood.

Example.—Blood is taken up to 0·5 mark.

Total number of cells counted in five groups of 16 small squares = 450.

Therefore 450 × 50 = 22,500 cells per c.mm. of diluted blood; and 22,500 × 200 = 4,500,000 cells per c.mm. of undiluted blood.

When, as in the above case, blood is taken to the 0·5 mark and the dilution is 1 in 200, the calculation can be simplified by adding four 0's to the total number of cells counted in the five small squares; e.g. in this instance 450. × 10,000 = 4,500,000.

For high counts, as in polycythæmia

One in 200 dilution is not enough as the cells are numerous and would be too crowded to allow an accurate count to be made, so the blood is drawn up to the 0·2 mark and the diluting fluid as before up to the 101 mark; this produces a dilution of 1 in 500, and the total number of cells in the five squares must be multiplied by 25,000 instead of 10,000 as in the example above.

Conversely, in an anæmic blood it is advisable to take blood up to the 1·0 mark; the dilution will be 1 in 100 and the total number of cells will be multiplied by 5,000.

A table (IV) which will facilitate calculation when blood is taken up to the different marks of the red cell pipette and cells from the five groups of small squares of the Neubauer rulings are counted, is given below.

TABLE IV

Blood taken	Dilution	Multiplication factor to arrive at number of cells per c.mm.
0·1 mark ..	1 : 1,000	× 50,000
0·2 „ ..	1 : 500	× 25,000
0·4 „ ..	1 : 250	× 12,500
0·5 „ ..	1 : 200	× 10,000
1·0 „ ..	1 : 100	× 5,000

The Thoma ruling.—The only way this ruling differs from that of the Neubauer is that with the Thoma ruling there are no separate squares for the white cell count, and that there is only one set of rulings instead of two, so that the count cannot be done in duplicate, unless the slide is washed, another drop of diluted blood added, and the coverslip re-applied.

Alternatively, but this does not constitute such a rigid check, a second set of five groups of squares can be counted on the same slide, without washing it and re-applying the coverslip.

As the area of the small square is the same as in the Neubauer hæmocytometer, i.e. $\frac{1}{400}$ square millimetre, the calculations are exactly the same as described above.

4

Enumeration of leucocytes

For enumeration of the leucocytes, follow the technique given under enumeration of erythrocytes, taking all the same precautions in the different steps.

With a dry white cell pipette suck blood up to the mark 0·5 (or 1 in cases of leucopænia), wipe off the blood adhering to the outside of the pipette, plunge it into the white cell diluting fluid and draw the mixture up to the mark 11, take off the tube and put a tightly fitting flat rubber band round to close both the ends, and lay it flat on the table.

Here, too, the capillary end up to the 1 mark of the pipette is occupied entirely by the diluting fluid. Hence the true dilution of the cells is 0·5 (or 1) in 10, i.e. 1 in 20 (or 1 in 10).

By taking blood up to the different marks from 0·1 to 1 in the pipette, dilutions ranging from 1 in 100 to 1 in 10 can be made.

Counting the cells and calculation to find out the cells per c.mm.

With Neubauer ruling.—Count the cells in the four corner areas A, B, C and D.

Divide by 4 to get the average number of cells in each area.

We know the area is 1 square millimetre and it is 0·1 millimetre deep.

Therefore the cubic capacity of each area is 0·1 cubic millimetre (1 × 0·1).

Thus, the cells counted are from 0·1 cubic millimetre of diluted blood.

This, when multiplied by 10, gives the number of cells per cubic millimetre of diluted blood. Again, this figure multiplied by the diluting factor 20 (or 10) gives the number of cells per cubic millimetre of whole blood.

Example.—Blood was taken up to 0·5 mark.

Number of cells counted from the four squares was 120.

Therefore, the average number of cells in 1 square, i.e. 0·1 cubic millimetre, of diluted blood is 30.

Therefore 300 (30 × 10) is the number of cells in 1 c.mm. of diluted blood;
and 6,000 (300 × 20) is the number of cells per c.mm. of blood.

The number of cells can be calculated rapidly by multiplying the total number of cells from the four squares by 50 $\left(\text{i.e. } \frac{20 \times 10}{4}\right)$ when the dilution is 1 in 20, or by 25 $\left(\text{i.e. } \frac{10 \times 10}{4}\right)$ when the dilution is 1 in 10.

Thus, in the above example,

120 = number of cells from the four squares.

Therefore 120×50 = 6,000 is the number of cells per c.mm. of blood.

For high counts, as in leukæmia.

In cases of leukæmia greater dilution of the blood is necessary for a correct count of the cells, as otherwise the cells are so crowded that an accurate count is

practically impossible. In such a case, blood is taken up to the 0·1 or 0·2 mark of a white cell pipette (or up to the 0·5 or 1·0 mark of a red cell pipette) and is filled up with white cell diluting fluid. The cells are counted from the four big squares as in the ordinary leucocyte count and the number of cells per cubic millimetre is calculated.

A table (V) for facilitating calculation when blood is taken up to the different marks of the pipette, and the cells in four areas of 1 square millimetre each are counted, is given below :—

TABLE V

Blood taken	Dilution	Multiplication factor to arrive at number of cells per c.mm.
0·5 in red cell pipette ..	1 : 200	× 500
1·0 „ „ „ „ ..	1 : 100	× 250
0·1 „ white „ „ ..	1 : 100	× 250
0·2 „ „ „ „ ..	1 : 50	× 125
0·4 „ „ „ „ ..	1 : 25	× 62·5
0·5 „ „ „ „ ..	1 : 20	× 50
1·0 „ „ „ „ ..	1 : 10	× 25

(A) WITH THOMA RULING : CALCULATION FOR LEUCOCYTES

All the leucocytes in the whole of the cross-ruled area are counted ; at least four separate counts should be made and the average count taken.

This area of 1 square millimetre represents a cubic capacity of 0·1 cubic millimetre. This when multiplied by 10 gives the number of cells per cubic millimetre of diluted blood and when again multiplied by the diluting factor (10 or 20) the number of cells per cubic millimetre of whole blood.

Thus, the number of cells multiplied by 100 (when the dilution is 1 : 10) or by 200 (when the dilution is 1 : 20) gives the number of cells per cubic millimetre of undiluted blood.

(B) COUNTING BY FIELD METHOD FOR LOW COUNTS

This method is suitable in kala-azar or other diseases in which there is usually a leucopænia. Take blood up to 0·5 mark of the white cell pipette and dilute it 1 in 20.

With 5 × eyepiece and one-sixth objective focus the square in the centre of the field of the counting chamber with the Thoma ruling, or the centre block of the Neubauer, and draw out the tube until the diameter of the field measures eight

times the length of the side of a small square. The area of the *whole field* is now equal to the area of 50 small squares almost exactly $\left(\pi r^2 = \frac{22}{7} \times 16 = 50{\cdot}2857\right)$.

Count the number of cells in 40 such different fields.

Calculation.—The volume of each square is 1/4,000 cubic millimetre and each field contains 50 squares.

Thus, if the total number of cells in 40 fields is 70, the number of cells per cubic millimetre of blood $= \frac{4{,}000 \times 20}{50 \times 40} \times 70 = 2{,}800$.

The same result may be obtained by multiplying the total number of cells in 40 fields by 40 if the dilution is 1 : 20, or by 20 if the dilution is 1 in 10.

With this method, cells of a very large area are counted and the multiplying factor is only 40, or 20, and therefore greater accuracy is ensured.

Enumeration of the nucleated cells of the marrow

The enumeration of the nucleated cells of the marrow from the material obtained by sternal biopsy is carried out in the same way as that of the leucocytes in cases of leukæmia.

Care of the instruments

After use, the pipettes, coverslip and the hæmocytometer chamber must be cleaned thoroughly.

Cleaning the pipettes

1. Thoroughly wash out all the diluted blood with water.
2. Remove water with absolute alcohol.
3. Remove alcohol with ether.
4. Pass air through to dry the pipette, so that the bead rolls freely in the bulb.

It is a very laborious process to clean the pipettes by sucking air through them, and one cannot dry them properly by blowing, as expired air is laden with moisture. The pipettes can be cleaned easily and dried by attaching them to a suction pump (figure 8), or when this is not available by attaching them to a syringe.

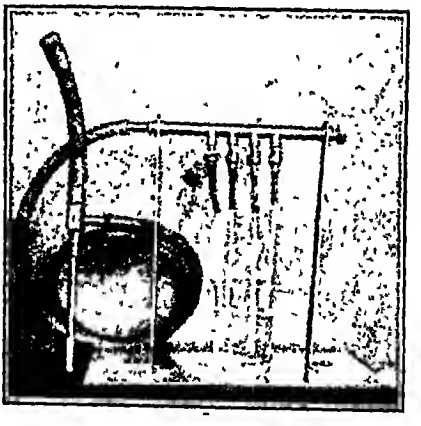


Fig. 8.—Suction apparatus for cleaning pipettes.

If the cells clog the tip or any part of the capillary tube, loosen them by inserting a stiff horse hair.

If there is albuminous matter in the bulb, fill it up either with saturated solution of NaOH or potassium bichromate cleaning solution, keep overnight in the 37°C. incubator, and clean next morning.

TABLE VI.

Showing the normal red cell counts of different populations

Sex	Age	Locality	Economic status	Number	Mean red cell per c.mm. in millions	Standard deviation	Authority
Males	19–30	Bombay	Students, etc.	121	5·110	± 0·380	Sokhey *et al.*, 1937.
	25–45	Calcutta	Mixed	50	5·362	± 0·633	Napier and Das Gupta, 1935*a*.
	20–45	,,	,,	30	5·533	± 0·490	,, ,, ,, 1936.
	19–30	Assam	Coolies	24	5·353	± 0·620	,, ,, ,, 1935*b*.
	19–30	,,	,,	20	5·270	± 0·710	,, ,, ,, 1936.
		Cachar	,,	25	5·057	± 0·563	Napier and Majumdar, 1938.
	Adults	U. S. A.	..	..	5·400	..	Castle and Minot, 1936.
	,,	Britain	..	..	5·690	..	Whitby and Britton, 1939.
	,,	,,	..	..	5·428	..	Price-Jones, 1931.
Females, non-pregnant	16–30	Bombay	Middle class	101	4·470	± 0·330	Sokhey *et al.*, 1938.
	14–38	Calcutta	..	125	4·615	± 0·409	Napier, Edwards and Das Gupta, 1941.
	17–30	Delhi	Middle class	101	4·560	± 0·250	Benjamin, 1939.
		Cachar	Coolies	25	4·454	± 0·705	Napier and Majumdar, 1938.
		Assam	,,	20	4·550	± 0·650	Napier and Bilimoria, 1937.
		Britain	..	..	4·800	..	Whitby and Britton, 1939.
		,,	..	..	5·012	..	Price-Jones, 1931.
	18–22	Michigan	Students	50	4·750	..	Bethel, 1936.
Females, pregnant ..	..	Assam	Coolies	40	4·650	± 0·620	Napier and Bilimoria (1937).
	..	Michigan	..	28	4·120	..	Bethel (1936).

Cleaning counting chamber and coverslip

Wash the ruled side of the counting chamber and the coverslip in running water. Thoroughly dry, first with a clean cotton handkerchief and finally with a silk handkerchief, selvyt cloth or lens paper, but avoid rubbing the ruled area of the counting chamber.

From time to time the counting chamber and the coverslip should be wiped with alcohol or acetone to remove any grease and then dried with the silk handkerchief, but on no account should the counting chamber be immersed or freely cleaned with alcohol.

If the rulings become faint after long use, the lines may be made prominent by lightly rubbing with a silk cloth on which graphite (lead from a pencil) has been rubbed.

Normal standards

The number of red cells per c.mm. is usually given in textbooks as 5,000,000. This figure is too low for men and too high for women. The mean of a number of counts in different populations is given in table VI.

It will be seen that there is a striking uniformity in the counts in different populations, compared for example with the hæmoglobin estimations in the same range of populations.

5

Enumeration of reticulocytes

Reticulocytes, or reticulated erythrocytes, are young red cells; they represent the stage just after the cells have lost their nuclei and before they are fully mature. The reticulocyte count is expressed as a percentage of the red cells; their percentage incidence gives information as to the activity of the red-blood-cell formation at the moment.

The reticulation may appear as a net-work over the whole cell, as a collection of discrete rods, or as granules. These are not shown by ordinary Romanowsky stains and a special supravital staining method is necessary to demonstrate the reticulation.

Staining methods.—Various dyes have been tried for staining reticulocytes, but brilliant cresyl blue has been found to be the best and it is now used universally. Although brilliant cresyl blue is the dye of choice, a number of different methods of using it have been suggested. Methods for reticulocyte staining may be classified in the following groups, in all of which

scrupulously clean and polished slides and coverslips without any scratches should be used*:—

A. Methods in which a thin film of the dye is dried on the slide or coverslip and the blood added later.

B. Methods in which the blood is mixed with a solution of the dye.

With either method (*i*) a dried smear, or (*ii*) a wet coverslip preparation may be made.

Apparatus required [for method of choice, B (ii)]

(*i*) Polished slides.
(*ii*) Clean coverslips (preferably 22 × 40 mm.).
(*iii*) Cresyl-blue solution 1 per cent in 0·85 per cent sodium chloride.
(*iv*) Capillary pipette.
(*v*) Rubber teat.
(*vi*) Vaseline or paraffin.
(*vii*) Gas or spirit lamp.
(*viii*) Ehrlich's eyepiece (or some means of limiting the microscopic field).
(*ix*) Microscope.

Additional requirements for other methods

(*i*) Surgical pricking needle or 'blood gun'.
(*ii*) Alcohol, ether, and cotton-wool.
(*iii*) Cresyl-blue solution 1 per cent in absolute alcohol.
(*iv*) Wide-mouthed glass bottle
(*v*) Specimen tube
(*vi*) Filter paper } A (*i*) and (*ii*).
(*vii*) Loishman's stain
(*viii*) Distilled water } A (*ii*) and B (*i*).

Method A

To prepare slides or coverslips proceed as follows:—

Make a 1 per cent solution of brilliant cresyl blue in absolute alcohol and keep it in a well-stoppered bottle. The slide may be prepared by one of the following methods:—

(*a*) Take a polished slide, heat it a little over a flame and put it on a flat even surface. With a capillary pipette take some brilliant cresyl-blue

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* *Slides.*—These should be cleaned and polished in the following way:—

(*i*) Only new slides should be used. Put the slides in a glass jar containing spirit. Keep them soaked in spirit for over 24 hours.

(*ii*) With a pair of forceps take out one slide at a time, allow the spirit to drain off the slide, and then flame it over a spirit lamp or gas burner.

(*iii*) Polish well one side of the slide with jeweller's rouge, using a soft cloth. Mark the polished side with a glass pencil and keep it in a dust-proof slide box.

Coverslips.—Keep the coverslips in a wide-mouthed glass jar soaked in spirit. Just before use, with a pair of fine forceps, take out one coverslip at a time, allow the spirit to drain off, flame them over a spirit lamp or gas burner, clean with a soft cloth and keep them covered in a small Petri dish until they are wanted.

solution from the stoppered phial and put a drop on the middle of the slide. If the slide is clean and it is on an even surface, the stain will spread concentrically and will be distributed in a uniform manner. The stain will dry in a minute or so; the slide is then ready for use.

(*b*) Put some stain in a wide-mouthed glass bottle. Warm a polished slide by passing it through a Bunsen flame, and holding it in a pair of forceps dip it vertically into the jar of the dye up to three-quarters of its length. Take it out, allow the excess of stain to drain back into the bottle, and, before it is dry, put it in an upright position inside a specimen tube at the bottom of which a piece of blotting paper has already been placed. Put in the cork and allow it to stay overnight; take it out, and wipe the stain from the unpolished side of the slide with a moist cloth; it is then ready for use.

Slides prepared in either of these ways may be kept for a long time in a dust-proof slide box and used when required.

(*i*) To prepare a dried smear, only slides prepared by the second method are suitable.

Put a drop of blood, oxalated or from the finger, on one end of a prepared slide and draw a very thin smear across the stained surface of the slide in the usual way—the use of a hæmocytometer coverslip as a spreader facilitates the drawing of a thin smear. Slides prepared in this way may be counterstained with any of the Romanowsky stains in the usual way.

(*ii*) To make wet coverslip preparations, slides prepared in either of the above methods may be used.

Put a drop of blood, oxalated or from the finger, on the stained surface of prepared slide, gently place a coverslip on the drop of blood, avoiding air bubbles as far as possible. If the drop of blood is of the proper size, it will spread uniformly under the pressure of the coverslip, but, in case it does not do so, apply very gentle pressure with the tip of the finger, so that the blood spreads evenly, but on no account should the coverslip be pressed hard, as some of the cells may be ruptured. Ring the coverslip round with hard paraffin by dipping a match in melted paraffin and stroking it on to the slide at the junction of the slide and coverslip.

Method B

In this method a small amount of blood is mixed with a quantity of dye in some definite proportion.

(*i*) Method of Osgood and Wilhelm (1934).

Put 0·5 c.cm. of 1 per cent brilliant cresyl-blue solution in 0·85 per cent sodium chloride into a small test-tube on a rack. Add an equal quantity of oxalated blood from a well-shaken flask. Mix well and allow the mixture to stand for about 2 minutes. Rotate the tube between the hands to get an even mixture.

Withdraw a little of the mixture with a capillary pipette and put a drop at one end of a polished slide. Make a thin smear and, when it is quite dry, counter-stain with Leishman's stain in the usual way. The slide is now ready for examination for reticulocytes. A differential count of the leucocytes can also be done at the same time.

(*ii*) Modification of the above method. *This is the method of choice.*

Prepare a mixture of cresyl blue and oxalated blood as in the above method. With a capillary pipette take out a little mixture and put a small drop at about the centre of the polished side of a clean slide. Apply a clean coverslip on the drop of the mixture, when, under the pressure of the coverslip, the mixture will spread evenly; but, in case it does not do so, very gentle pressure with the tip of the finger may be applied over the coverslip. The preparation is then sealed with vaseline or paraffin and is ready for examination for reticulocytes only.

Counting the reticulocytes

Slides prepared by any of the methods described above are first examined rapidly with a low power (two-thirds objective), and a portion of the slide is selected where the cells appear discrete and there are not too many in one field. This portion of the slide is now examined with the one-twelfth oil-immersion lens. The total number of red cells in the field and also the number showing reticulation are counted. The use of an Ehrlich's eyepiece, or a piece of metal with a square hole in it placed inside the eyepiece to narrow down the field, greatly facilitates the counting. Altogether one thousand red cells are counted in a more-or-less normal case, but if the count is high, 5 per cent or more, 500 red cells are sufficient; the number of cells showing reticulation is noted, and from this the number of reticulocytes per 100 red cells is calculated.

A Veeder counter, or some similar device, is useful here; one counts the red cells, and each time a reticulocyte is encountered clicks the Veeder. When a thousand red cells have been counted, the number on the Veeder is read.

In the absence of an automatic counter, a pencil and paper must be used, for it is a mistake to try to keep two sets of figures in one's head.

Comment

Osgood and others have shown that divergent results are obtained with the different methods in use. They obtained the best results with the method described by them, which has the additional advantage that a differential count of the leucocytes can also be done in the same preparation. We have, however, found that the modified Osgood method, which is the routine procedure in our laboratory, has given even better results in our hands than the original method. With the modified method all the reticulocytes stain very well and there is no clumping nor overlapping of the cells; thus, the counting is very much facilitated. With the original method a number of cells appear broken, more so in the case of anæmic blood, and the cells

are less discrete. The percentage of reticulocytes appears to be slightly lower in the original than in the modified method. In a small number of cases where the counts were done by both methods, 5·16 per cent reticulocytes were found by the original method, against 5·98 by the modified method.

On account of the divergent results obtained by the different methods in use, for comparative studies a uniform technique should be adhered to in the enumeration of the reticulocytes, not only by one group of workers, but by all, so that the results of different workers will be comparable.

The modified Osgood method, described above, is simple and, as it appears to be superior to the other methods in many ways, we recommend it as the method of choice for the enumeration of reticulocytes.

Normal values

It is unnecessary to quote the figures of numerous observers regarding the normal percentage of reticulocytes. When, in a healthy individual whose red cells are at the normal physiological level, the hæmopoietic and hæmolytic tissues are functioning normally, the number of reticulated red cells reaching the peripheral blood is less than 1 per cent of the total red cells. The normal range is usually given as 0·1 to 1·0 per cent. The present writers found a mean percentage of 0·67 ± 0·37 in a series of 50 city-dwelling Indian males, with a range from 0·2 to 1·4, and 0·37 ± 0·27 amongst 122 women. On the other hand, for Assam tea-garden coolies the mean was 2·17 ± 1·92 and the range 0·1 to 10·8 per cent. It is probably true to say that reticulocyte counts in man of over 1·0 per cent are evidence of some unusual stimulation to the hæmopoietic system, though they may be encountered in an apparently healthy individual. In our Assam coolie series, we accepted these findings of unusually high reticulocyte percentages, associated as they were with a low hæmoglobin percentage, as evidence of some abnormality of the erythron in the individuals concerned.

Significance of reticulocytes

Whenever the hæmopoietic system meets an extraordinary demand for red blood cells, the reticulocyte percentage will rise, but the reticulocyte rise will *not* be maintained even though it will take some time for the deficiency of the red cells to be made good.

For example, if a healthy individual loses a large quantity of blood, there is an immediate steady rise in the reticulocyte curve which reaches its maximum and falls to normal again within a few days, whereas it will probably be a few weeks before the loss of blood cells is made up.

Similarly, in deficiency anæmias, when the deficient substances, e.g. hæmopoietin in pernicious anæmia and iron in iron-deficiency anæmia, are supplied, the hæmopoietic tissues in the bone marrow are now enabled to meet the demand for red cells, and there is a rise in the reticulocyte percentage that starts within two or

three days and reaches its maximum within five to ten days, after which it falls as fast as it rose.

This reticulocyte rise gives a valuable indication that a deficiency has been made good.

A third example is in toxic aplastic anæmia, where a toxin is depressing the function of the bone marrow. In this case there may be no reticulocytes at all to be found in the peripheral blood, but once the toxin is neutralized, or otherwise ceases to exert its toxic effect, the hæmopoietic tissues will start to function properly, reticulocytes will appear in the blood, their percentage will rise to a maximum within a few days and will fall again to the normal level, but in the absence of any further intoxication the red cells will continue to increase until they reach their normal level.

It should be emphasized that whilst a reticulocyte crisis, as this sharp rise is called, is evidence that the hæmopoietic tissues are functioning effectively, a fall of reticulocytes to the normal level does not indicate any cessation of this functioning, but on the contrary it usually indicates that red blood cell formation is being carried on in an orderly manner.

In true hæmolytic anæmias, there is a constant reticulocytosis, as not only will the anoxæmia stimulate erythrogenesis, but there is a second source of stimulation in the debris of abnormal hæmolysis which has to be disposed of by the reticulo-endothelial tissues.

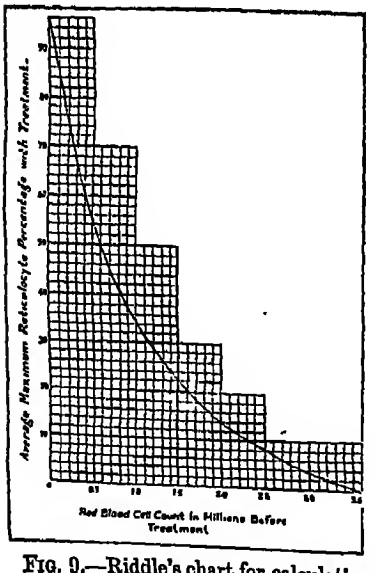


FIG. 9.—Riddle's chart for calculating the maximum reticulocyte response expected in pernicious anæmia with adequate therapy.

A constant reticulocyte count of 5 to 10 per cent or higher is evidence of excessive hæmolysis, and in a hæmolytic anæmia the return of the reticulocyte count to normal is usually evidence that the excessive blood destruction has ceased.

Reticulocyte crises

The extent of the reticulocyte response is governed mainly by the original level of the red cells in the anæmic state. For example, in a case of pernicious anæmia the rise in the reticulocyte percentage after the same adequate dose of liver extract may be as high as 55 per cent or practically negligible, according to whether the red cell count was 500,000 per c.mm. or 3,000,000 per c.mm. before treatment. Doubling the dose of liver extract would make no difference to the height of the reticulocyte response, but reducing it to an *in*adequate dose would. Therefore, in pernicious anæmia, if one has a chart showing the expected reticulocyte response, one can

judge whether or not the dose of liver extract given was an adequate dose. We have given a chart here, not so much for practical use as pernicious anæmia is rare in India, but to demonstrate this point.

In iron-deficiency anæmia, the response to adequate iron therapy is also proportionate to the degree of the anæmia, but in this case the reticulocyte count does not respond with the same mathematical precision.

6

Enumeration of thrombocytes

Thrombocytes, or blood platelets, are colourless or slightly bluish bodies, spherical, ovoid, or pear-shaped, and are usually about one-third of the diameter of a red blood cell, but they may be larger, especially in pathological conditions.

No method of enumeration of blood platelets is entirely satisfactory, as platelets tend to form clumps and to stick to any foreign surface with which they may come into contact. However, with careful technique, counts sufficiently accurate for practical purposes are not difficult to obtain. The principal point to be remembered in the platelet count is that manipulation of the sample of blood should be avoided, and, if possible, the blood should be taken directly on to the counting slide.

The platelet count is expressed as a number per cubic millimetre of blood. There are several methods for estimating the number of platelets; these fall under the two main heads, the direct and indirect methods.

Apparatus required [for method of choice, B (i)]

As in the reticulocyte count.

Additional requirements for other methods

For method A.—(*i*) Red blood cell pipette.

(*ii*) Sodium citrate 2 per cent solution (fresh and sterilized).

(*iii*) Counting chamber with Neubauer ruling and coverslip.

For method B.—(*ii*) Magnesium sulphate 14 per cent solution.

A. *Direct method.*—In this method the number of platelets per cubic millimetre is calculated without any reference to the red blood cell count.

Prick the finger with a sharp surgical needle and with a red cell pipette draw blood up to the 0·5 mark and dilute it with fresh sterile 2 per cent solution of sodium citrate, up to the 101 mark. After gentle shaking, put a drop in the counting chamber, wait 3 to 4 minutes to allow the platelets to settle down, focus the light carefully, and count with the high power all the platelets in 1 square millimetre area. The number of platelets per cubic millimetre of blood is then calculated in the usual way.

B. *Indirect method.*—As in the enumeration of the reticulocytes, the platelets are at first expressed as a percentage of the red cells calculated from the number of platelets observed in counting one thousand red cells. The number of platelets per cubic millimetre can then be calculated from the total red cell count, which must be done at the same time. Of the many indirect methods advocated, the following two methods have given consistently good results in our hands :—

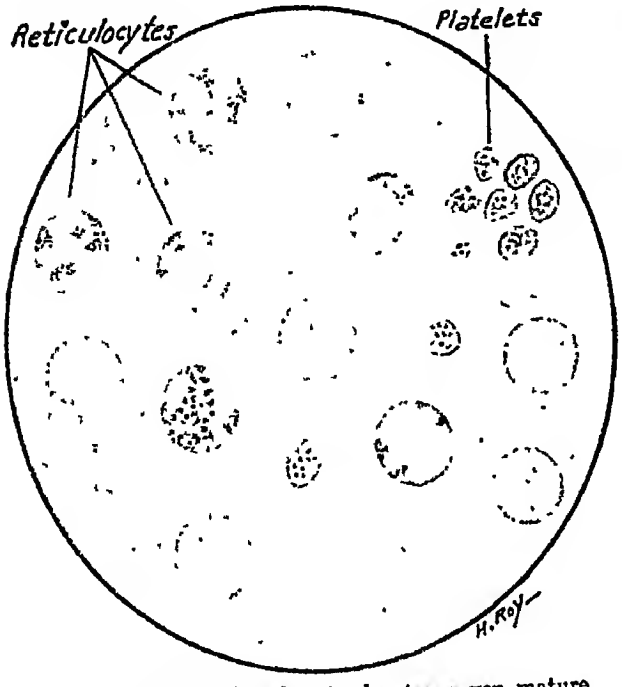


FIG. 10.—Showing six reticulocytes, seven mature erythrocytes, and nine platelets.

(*i*) Clean the tip of the finger first with soap and water, and then with alcohol and ether, and apply a thin layer of sterilized vaseline over it. Prick the finger with a blood gun or sharp pricking needle through the thin layer of vaseline. As the blood comes out, touch the uppermost portion of the blood drop with the centre of a 22 × 40 mm. coverslip. Place the coverslip on a dried cresyl-blue-stained slide prepared by method A, (*a*) or (*b*), as described for the reticulocyte count. With very gentle pressure try to obtain as uniformly spread a film as possible, and finally seal the sides of the coverglass with vaseline or paraffin.

(*ii*) Clean the tip of the finger as above. Balance a drop of 14 per cent solution of magnesium sulphate on the back of the finger just above and to one side of the nail groove. Make a small puncture through the drop. The drop of blood should be such that the resultant blood mixture is about 1 in 20. Mix rapidly with a paraffined* capillary pipette. Transfer the blood mixture to clean slides, make very thin smears and stain with Wright's or Leishman's stain.

The coverslip preparation (*i*), or the slide (*ii*), is examined with an oil-immersion lens. A small rectangular field is more convenient for counting than a big circular one (*v. s.*). Note the number of platelets seen in counting 1,000 red cells.

Successive fields from the different areas of the smear should be examined so as to eliminate, as far as possible, the error from uneven distribution.

* Platelets do not adhere to a paraffined surface, so that if glass is covered with a thin layer of paraffin the platelets do not tend to adhere to it. The pipette is prepared by drawing hot melted paraffin wax through it ; a thin layer of paraffin will adhere to the glass when it cools.

The red cell count is done as a separate procedure, in the usual way.

By a simple calculation the total number of platelets per c.mm. may now be ascertained. Thus, if the total red cell count is 5,000,000 per c.mm., and if x be the number of platelets counted against 1,000 red cells then $\frac{x \times 5,000,000}{1,000}$ or $x \times 5,000$ is the total number of platelets per c.mm.

Example.—Total red blood cells = 4,500,000 per c.mm.
Number of platelets counted against 1,000 red cells = 50.
Therefore,
platelets per c.mm. $= \frac{50 \times 4,500,000}{1,000}$ or $50 \times 4,500 = 225,000$.

The enumeration of the platelets by the indirect method, though more laborious and time absorbing, as a total red cell count has also to be made, is nevertheless much superior to the direct method. Results obtained by indirect methods, especially by method (*i*), are always higher than those of the direct method, as there is very little contact with foreign surfaces.

Normal values

The figures given by different writers vary considerably. This variation is undoubtedly due to some extent to the differences in the methods adopted for counting them, but there are considerable variations in the platelet counts done at different times by the same method in the same individual.

Whitby and Britton (1939) in Great Britain give the range as 250,000 to 500,000 per cubic millimetre and Gram (1920) in America as 280,000 to 540,000 with the average at 350,000. The present writers found 369,000 ± 248,000 in Calcutta and 423,000 ± 343,000 in Assam; in the latter series there were a number of so-called normal individuals with platelet counts of over a million and a half.

7

Determination of corpuscular volume

Principle.—A volume of uncoagulated blood is centrifuged at a high speed in a graduated tube until there is no further packing of the corpuscles. The level of packed red cells and of the serum are read off directly from the tube; from this the proportion of the packed cells to the whole blood is calculated and is expressed as a percentage.

Apparatus required

(*i*) Graduated cell-volume tubes fitted with rubber corks.
(*ii*) Capillary pipettes with rubber teats.
(*iii*) High-speed centrifuge machine that will rotate at at least 3,000 revolutions per minute.
(*iv*) A balance to ensure that the weight of the tubes and their contents are about equal.
(*v*) Hand lens.

Cell-volume tubes.—Various types of tube are in use; these vary in length, bore, and capacity. Wintrobe uses small tubes, 11 centimetres long with a bore

of 2·5 millimetres, which are marked in millimetres and centimetres from 0 to 10. In our laboratory, we use a tube 7·5 centimetres long with a bore of 7 millimetres and graduated from 0 to 110. These tubes were specially made for us, by Messrs. Baird and Tatlock, London, to fit in our high-speed centrifuge machine, but comparatively short graduated tubes of this kind are now easily obtainable from instrument makers.

As the degree of packing of the cells depends to a certain extent on the calibre of the tube, it is desirable that the same set of tubes which were used in working out the normal standards should be used in all subsequent work.

Method.—The blood is taken in the usual way into a 5-c.cm. or 3-c.cm. flask containing oxalate (*see* p. 36). With a capillary pipette withdraw oxalated blood from the flask after it has been well shaken, fill up the graduated cell-volume tube from the bottom upwards exactly up to the 100 mark, put in a small rubber cork to prevent any evaporation during centrifugalization, and place it in the centrifuge bucket.

If possible two specimens should be placed in the centrifuge at the same time. In any case, the centrifuge bucket containing the graduated tube must be balanced against a similar bucket and tube so that their weights are exactly equal, before these two buckets are placed in opposite sides of the centrifuge; this elementary principle must be observed rigidly, both for the sake of the centrifuge and for satisfactory packing. Centrifuge at a high speed—2,500 to 3,000 revolutions per minute—until there is no further packing of the red cells.

The maximum time required for complete packing of the red cells must be found out by a few preliminary experiments. This will depend to a great extent on the centrifuge machine.

The tubes are first spun for 20 minutes at a high speed, the actual speed being noted; they are taken out and the readings taken; they are again spun for another 5 minutes and the readings again taken. The process is continued until there is no difference between two consecutive readings—the time at which the first of these two readings was taken is the time required for complete packing of the cells as there was no further packing. The mean time in minutes for 10 to 20 such experiments is taken as the time required for complete packing of cells with that particular centrifuge machine at that particular speed. However, in all subsequent examinations a little extra time, say 5 minutes, should be allowed over and above the mean time determined.

Generally, the cells are fully packed if the tubes are spun for about 20 minutes in a centrifuge machine at a speed of 2,500 to 3,000 revolutions per minute, but it is safer to allow 30 minutes.

The tubes are now taken out of the buckets and with a hand lens the marking on the tube corresponding to the level of the packed red cells is noted; also the top level of the serum is checked. If this is at the mark 100, then

the red cell reading can be translated directly into a percentage, but if it is more or less than 100 the percentage figure must be calculated making an allowance for this fact.

Example.—If the top of the serum is at the 102 level and the red cells at 42, the cell volume is $\frac{100}{102} \times 42 = 41{\cdot}17$ per cent.

With leukæmic blood it is almost impossible to read the top level of the red cells accurately and therefore it is difficult to ascertain the correct cell volume.

Anti-coagulant.—This has been discussed above (p. 36), but perhaps we should again mention that, if the isotonic mixture of potassium and ammonium, which we recommended, be used, no shrinkage will occur.

On the other hand, if potassium oxalate alone (0·2 per cent) be used, then a factor, × 1·09, must be applied to the cell volume before the corpuscular values are calculated.

Example.—The uncorrected cell volume is 44 per cent; the corrected cell volume will be $44 \times 1{\cdot}09 = 47{\cdot}96$.

Calculation of corpuscular values

From the red cell count, the hæmoglobin expressed in grammes per cent, and the corpuscular volume, certain absolute values can be calculated.

These values are :—

(1) the mean corpuscular volume (MCV),

(2) the mean corpuscular hæmoglobin (MCH), and

(3) the mean corpuscular hæmoglobin concentration (MCHC).

Mean corpuscular volume

This is the mean, or average, volume of a single red cell.

It is expressed in cubic microns (cu.μ) and is obtained by the following method :—

$$\text{MCV} = \frac{\text{Volume of packed red cells in c.cm. per 1,000 c.cm. of blood}}{\text{Red cells in millions per c.mm.}}$$

Example.—Corpuscular volume = 40 c.cm. per 100 c.cm. of blood.

Red cells count = 5,000,000 per c.cm.

Mean corpuscular volume = $\frac{40 \times 10}{5} = 80{\cdot}0$ cubic microns (cu.μ).

Mean corpuscular hæmoglobin

This is the average hæmoglobin content of a single red cell expressed in micro-microgrammes ($\gamma\gamma$).

It is obtained by the following method:—

$$\text{MCH} = \frac{\text{Hæmoglobin in grammes per 1,000 c.cm. of blood}}{\text{Red cells in millions per c.mm.}}$$

Example.—Hæmoglobin = 14 grammes per 100 c.cm. of blood.
Red cells = 5,000,000 per c.mm.

$$\text{Mean corpuscular hæmoglobin} = \frac{14 \times 10}{5} = 28{\cdot}0\gamma\gamma.$$

Mean corpuscular hæmoglobin concentration

This is the mean, or average, of the hæmoglobin concentration in each cell, and is expressed as a percentage of the cell contents:—

$$\text{MCHC} = \frac{\text{Hæmoglobin in grammes per 100 c.cm. of blood}}{\text{Volume of packed cells in c.cm. per 100 c.cm. of blood}} \times 100.$$

Example.—Hæmoglobin = 14 grammes per 100 c.cm. of blood.
Corpuscular volume = 40 c.cm.

$$\text{Mean corpuscular hæmoglobin concentration} = \frac{14}{40} \times 100 = 35{\cdot}0 \text{ per cent.}$$

That is to say, in this instance 35 per cent of the cell substance consists of hæmoglobin.

The great advantage of these mean values is that, in calculating them, it is not necessary to decide on any arbitrary normal, as one does in calculating indices, e.g. the colour index, for arriving at which one usually considers 5,000,000 red cells per c.mm. and 100 per cent hæmoglobin to be the normal values. Unqualified, a colour index is a meaningless expression as even if the above normal values have been taken, it is still uncertain what 100 per cent hæmoglobin means (*vide* p. 39). If *later* one decides on the normal values for the particular population, then it is a very simple matter to calculate the colour index.

Example.—MCH of the particular case = 35$\gamma\gamma$.
Mean MCH of the population = 30$\gamma\gamma$.

$$\text{Colour index (CI)} = \frac{35}{30} = 1{\cdot}1\dot{6} = 1{\cdot}17.$$

Similarly, the volume index and the saturation index can be calculated from the MCV and MCHC, respectively.

There is in our opinion little point in calculating these indices, and we recommend workers to get into the habit of thinking in terms of mean corpuscular values instead of indices, just as they should get into the way of thinking in terms of grammes of hæmoglobin instead of percentages.

Normal values.—There are undoubtedly different normal values for different populations. We will consider mean corpuscular volume first: Whitby and Britton give 86 as the mean, with the range from 80 to 94, but neither these writers, nor the majority of others who give a 'normal range', make any attempt to explain what they mean by the 'normal range'. They can scarcely mean that *all* normal observations fall within the range, but they do not specify what percentage of observations may be expected to fall within it. If one gives the mean (m) and the

Table VII

Corpuscular values

Sex	Locality	Number	Mean corpuscular volume	Standard deviation	Mean corpuscular hæmoglobin	Standard deviation	Mean corpuscular hæmoglobin concentration	Standard deviation	Authority
Male ..	Calcutta	30	90·49	± 7·90	28·53	± 2·31	31·07	± 1·20	Napier and Das Gupta, 1936.
,, ..	Bombay	121	87·08*	..	30·01	..	34·54*	..	Sokhey *et al.*, 1937.
,, ..	Assam	24	71·29	± 7·04	23·93	± 2·31	32·50	± 3·10	Napier and Das Gupta, 1936.
,, ..	Cachar	25	84·93	±10·78	25·14	± 3·70	29·72	± 2·94	Napier and Majumdar, 1938.
Unspecified ..	U. S. A.	..	87·00	..	27·5	..	35·00	..	Castle and Minot, 1936.
,, ..	Britain	..	86·00	..	29·5	..	34·00	..	Whitby and Britton, 1939.
Females ..	Calcutta	128	86·82	± 7·28	27·42	± 2·89	31·57	± 1·76	Napier, 1939.
,, ..	Bombay	101	88·53*	..	29·06	..	32·86*	..	Sokhey *et al.*, 1938.
,, ..	Delhi	100	92·7 †	..	28·76	..	33·58	..	Benjamin, 1939.
,, ..	Assam	20	77·30	± 7·70	24·50	± 3·00	31·20	± 1·70	Napier and Bilimoria, 1937.
,, ..	Cachar	25	82·49	±12·68	23·42	± 3·10	28·67	± 3·37	Napier and Majumdar, 1938.
,, ..	Michigan	50	86·30	..	..	..	..	..	Bethel, 1936.
Pregnant females	Calcutta	64	86·83	±10·8	26·62	± 3·34	30·57	± 2·13	Napier *et al.* (unpublished).
,, ,,	Assam	40	72·10	..	23·80	..	32·60	..	Napier and Bilimoria, 1937.
,, ,,	Michigan	28	92·00	..	..	..	..	..	Bethel, 1936.

* Calculated from data given, after applying factor × 1·09 to cell volume to allow for shrinkage, where this has not been done.

† Corrected figure recently supplied by worker.

standard deviation (sd) of a number of observations, that at least does mean something definite, namely that two-thirds of the observations will fall between m + sd and m — sd, and that 19 out of 20 will fall between m + 2 × sd and m — 2 × sd.

The last figures seem reasonable ones for practical purposes and can certainly be applied amongst hæmopoietically more stable populations than we usually encounter in India, but in most of our populations it provides too wide a range. Even for our Calcutta series in which the mean is 90·49 cu.μ, the range would be (90·49 + 2 × 7·90 =) 106·29 to (90·49 — 2 × 7·90 =) 74·69, or in round figures from 75 to 106: this is too wide to be considered a normal range, though the mean is close to that given by other workers. On the other hand, some of the Assam, so-called normal, populations have a very low MCV, and if the same rule were applied the range would be ridiculously low, e.g. 57·21 to 85·37 cu.μ in the first Assam series quoted, but there is evidence in this case that the whole population is suffering from a degree of iron-deficiency anæmia; therefore, as the figures are not based on a truly normal population, this rule cannot be applied.

It is obvious that no hard and fast rule can be laid down. Whenever possible, it is best to examine normal individuals of the population concerned, and, where one can be certain of excluding most of the cases of sub-clinical blood dyscrasia, to calculate the range from the mean *plus* or *minus* twice the standard deviation. Otherwise, 80 to 100 cu.μ may be taken as the normal range for general use in India. That is to say, any figure below 80 cu.μ may be taken as indicating microcytosis and any above 100 cu.μ as indicating macrocytosis.

Similarly, for the mean corpuscular hæmoglobin, on the Calcutta figures the normal range should be 28·53 ± 2 × 2·31 = 23·91 and 33·14, or roughly 24 to 33$\gamma\gamma$. This is in our opinion a good normal range for general use in India; any figure below 24$\gamma\gamma$ indicates hypochromia and any above 33$\gamma\gamma$ hyperchromia.

Finally, a good range for the mean corpuscular hæmoglobin concentration is 30 to 35; this is not based on any of the data quoted, for reasons into which we need not go now, but on our general experience.

8

Hyperbilirubinaemia and van den Bergh's test

Bilirubin is normally present in small quantities in blood plasma and blood serum; their yellow colour is in part due to this substance.

Ehrlich observed that when sulphanilic acid and sodium nitrite were added to a solution of bilirubin, a bluish-violet substance, azo-bilirubin, was formed. The reaction is specific and very delicate; will show bilirubin in a dilution of 1 in 1,500,000.

Van den Bergh made use of these observations, not only to test for the presence of an excess of bilirubin, but to draw certain conclusions from the nature of the

result. His original claims are now questioned, but his general conclusions hold good and form the basis of the test which we are describing.

Bilirubin is a by-product of hæmolysis. It is normally present in the blood in a dilution of about 1 in 400,000 or 0·25 mg. in 100 c.cm. of blood. It is excreted *via* the bile ducts after passage through the parenchyma cells of the liver; during its passage through the liver cells the blood bilirubin loses its protein molecule. If the passage of the bile is obstructed (obstructive jaundice), the bilirubin from the bile canaliculi is reabsorbed into the blood stream; this bilirubin, stripped of its protein molecule, reacts readily with the diazo reagent and gives what is known as a *direct reaction*.

In conditions of excessive hæmolysis and/or liver dysfunction, the amount of bilirubin present in the blood naturally increases; the diazo reagent does not react with this blood bilirubin immediately but only after it has been in contact with it for some time—this is sometimes known as a *delayed direct reaction*, but this reaction is not usually taken into consideration as it has no special significance beyond that covered by the indirect reaction which is clearer cut and susceptible to quantitative appraisement (*v. i.*). However, if the serum is first treated with alcohol and ammonium sulphate, the protein is precipitated and then an immediate reaction occurs; this is known as the *indirect reaction*.

A third type of reaction is that in which there is an immediate faint colour which steadily increases, indicating that both forms of bilirubin are present; this is known as the *biphasic reaction*.

The technical details of the test have been modified many times and it is done in a number of ways in different laboratories. Godfried (1935) recommends the method described by Thannhauser and Andersen (1921) as the most suitable for clinical purposes; this is the method that we follow in our laboratory.

Apparatus required

(*i*) Graduated pipettes, 1 c.cm., 2 c.cm., 5 c.cm., and 25 c.cm.
(*ii*) Small test-tubes, 4 inches by ½ inch.
(*iii*) Centrifuge tubes.
(*iv*) Small glass beakers or flasks.
(*v*) Capillary pipettes with rubber teats.
(*vi*) Centrifuge machine.
(*vii*) Sealed cobalt sulphate standard tubes.
(*viii*) * A Lovibond comparator with the coloured discs for bilirubin.

Reagents

Alcohol—absolute and 90 per cent.

Saturated solution of ammonium sulphate; this is prepared by dissolving an excess of ammonium sulphate in hot water (so that some undissolved salt is left at the bottom).

Normal saline.

* The comparator can be obtained from British Drug Houses, London, but stocks are now available at this company's depots in India, Imperial Chemical House, Ballard Estate, Bombay, or c/o H. S. Clark, Kent House, 33, Mission Row, Calcutta.

Diazo reagent.
(A) Sulphanilic acid—1 gramme.
Concentrated hydrochloric acid—15 c.cm.
Distilled water 1,000 c.cm.
(B) Sodium nitrite—0·5 gm.
Distilled water 100 c.cm.

Stock solutions of (A) and (B) are made. Separately, they keep well for a long time.

The test can be done either with the serum or plasma. When the cell volume is also being estimated, the supernatant plasma in the cell-volume tubes, after the packing of the red cells, is quite sufficient for the test. When the test is done by itself, 3 cubic centimetres of blood is drawn and placed in a dry test-tube or an oxalated test-tube.

The blood is collected in the usual way, and even the slightest hæmolysis must be avoided (*vide supra*). Preferably, the blood should be collected in the fasting state as it is claimed that food intake, especially a big meal of carbohydrate and fat, affects the level of serum bilirubin. The specimen must always be examined within 2 hours, as otherwise paradoxical results may be obtained.

Technique.—Prepare the diazo reagent by mixing 10 c.cm. of (A) and 0·3 c.cm. of (B) in a small flask or beaker.

This mixture must be prepared just before use and must never be used later than one hour after it is made.

Pipette off the clear supernatant plasma from the cell-volume tubes.

Direct reaction.—Take exactly 1 c.cm. of plasma in a centrifuge tube.

Add exactly 0·5 c.cm. of diazo reagent, and mix well.

One of the following things may happen:—

(*i*) Direct reaction—The mixture becomes reddish-violet within 30 seconds.

(*ii*) Biphasic reaction—The mixture becomes slightly reddish at once and the colour gradually increases in intensity and becomes reddish-violet.

(*iii*) Negative result—No change of colour occurs within 10 minutes.

Indirect reaction.—If no change occurs (*iii*), add to the mixture of plasma and diazo reagent 2·5 c.cm. of alcohol (absolute or 96 per cent), and 1·0 c.cm. of saturated solution of ammonium sulphate.

Mix well by inverting the tube, allow it to stand for about 2 minutes, and centrifuge for 5 to 10 minutes.

A positive indirect reaction is indicated by the supernatant fluid becoming coloured a distinct purple, the intensity depending on the amount of bilirubin present. As bilirubin is present in normal blood, a faint violet colour nearly always appears; such a reaction obviously has no pathological significance.

Quantitative indirect reaction.—It will be clear that some form of quantitative estimation is essential. Many elaborate methods which require special apparatus have been devised, but a fairly accurate estimation of the bilirubin content can be obtained by carrying out the test in the way described above and comparing the supernatant fluid with a prepared standard or with a permanent colour standard.

(*i*) Comparing the supernatant coloured fluid with the cobalt sulphate standard (2·161 per cent) has given the most satisfactory colour matching in our hands.

Preparation of the cobalt standard solution.—Dissolve 2·161 grammes of anhydrous cobaltous sulphate in 100 c.cm. of distilled water. The colour of this solution corresponds to that of 1 in 200,000 bilirubin or 0·5 mg. of bilirubin per 100 c.cm. Solutions equivalent to 0·4, 0·3, 0·2, 0·1 and 0·05 mg. per 100 c.cm. are made by diluting the original solution.

About 2 c.cm. of each of these solutions is put into tubes made of hard glass. The tubes are then sealed, numbered, and kept in the dark, and are brought out only when required. Properly stored, the solutions retain their colour for a fairly long time. In our laboratory, we did not find any difference in colour in solutions which were in daily use for over six months, when these were compared with freshly prepared solutions.

Technique for the use of cobalt standard solutions.—Take about 2 c.cm. of the coloured supernatant fluid in a tube having identically the same bore as the standard tubes, and compare its colour in a comparator with the cobalt standard tubes. The figure on the tube giving the correct matching with the supernatant fluid multiplied by four gives the bilirubin value of the undiluted serum, for the dilution of the serum in the supernatant fluid containing azo-bilirubin is 1 in 4 and not 1 in 5, because the saturated ammonium sulphate remains as a separate layer and does not contain azo-bilirubin.

If the supernatant fluid is of a deeper colour than the cobalt standard tube marked 0·5, dilute the supernatant solution with normal saline (dilution with 67 per cent alcohol which is recommended makes the solution hazy and difficult to match) until it matches one of the cobalt standard tubes. Note the dilution required to match and calculate the amount of bilirubin in the specimen.

Example.—One cubic centimetre of supernatant fluid was taken, to which was added 1 cubic centimetre of normal saline to match the tube marked 0·4.

First dilution of serum in the supernatant fluid is 1 in 4.

Second dilution to match the colour is 1 in 2.

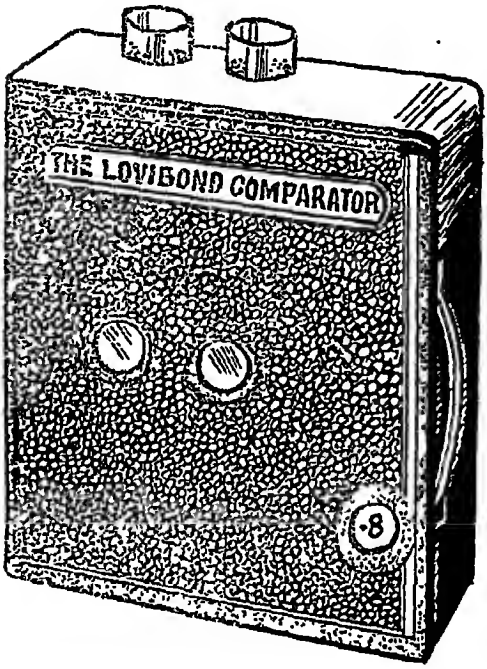
Therefore, total dilution is $\frac{1}{4} \times \frac{1}{2} = \frac{1}{8}$.

Then the original plasma contained $0{\cdot}4 \times 8 = 3{\cdot}2$ mg. of bilirubin per 100 c.cm.

(*ii*) *Lovibond comparator.*—Quantitative estimation of the indirect van den Bergh reaction can be simplified by the use of a bilirubin disc in the Lovibond comparator. The disc contains coloured glass standards marked 0·2, 0·4, 0·6, 0·8, 1·0, 1·25, 1·50, 1·75 and 2·0 mg. which are calibrated in such a way that, when the test

is done in the way described above, the matching with any of the discs gives directly the result for the undiluted serum (figure 11).

Technique for the use of the comparator.—Fit in the disc marked 'bilirubin' in the Lovibond comparator. Transfer the coloured supernatant fluid to the right-hand tube of the comparator. A tube of distilled water should be put behind the hole showing the coloured glass. Hold the comparator against a source of light (daylight is much better than any artificial light). Rotate the disc till a correct



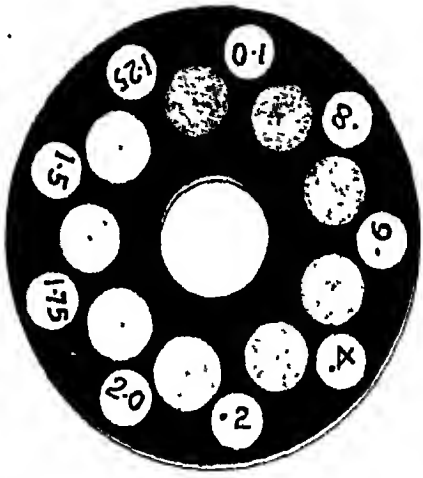


FIG. 11.

Lovibond comparator with the bilirubin disc and tubes in position.

Bilirubin disc for the Lovibond comparator.

colour match is obtained; the number that is visible indicates the bilirubin value in mg. per 100 c.cm. of undiluted serum or plasma, e.g. 0·8 mg. in the above figure.

When the colour of the supernatant fluid appears to lie between two consecutive coloured discs and does not exactly match either of them an intermediate value is recorded.

If the bilirubin value exceeds 2 mg. per 100 c.cm., dilute the supernatant fluid with normal saline until a correct match is obtained with one of the coloured glasses. Calculate the bilirubin value by multiplying the number on the disc by the dilution factor.

TABLE VIII

Showing the normal bilirubin, according to different observers

Author	Bilirubin in milligrammes per 100 c.cm. of plasma or serum		
	Range	Mean	S. D.
van den Bergh (Vaughan and Haslewood, 1938).	0·08–0·24	..	..
Greene *et al.* (1925)	0·3–2·0	..	..
Perkin (1927)	0·05–0·35	..	..
Barron (1931)	0·1–0·24	..	..
Elton (Vaughan and Haslewood, 1938) ..	0·0–0·25	..	..
Vaughan and Haslewood (1938) ..	0·2–1·7	0·54	± 0·25
Mills and Mawson (1938)	0·1–1·0	0·31	± 0·16
Napier and Das Gupta (1941) ..	0·0–1·0	0·20	± 0·23

Icterus index

Like the van den Bergh reaction, the icterus index is a measure of the bilirubin content of the blood. Though the test is simple to carry out, it does not give all the information which is obtained by the van den Bergh test.

Principle of the test.—The colour of the serum or plasma is compared with a standard solution of 1 in 10,000 potassium bichromate, which is taken as the unit.

Apparatus required

(*i*) Test-tubes in a rack.
(*ii*) Capillary pipettes with rubber teats.
(*iii*) Potassium bichromate solution 1 in 10,000 (to be stored in the dark).
(*iv*) Colorimeter of the Duboseq or Klett type.

Technique.—Pipette off the supernatant plasma from the cell-volume tube after complete packing of the cells.

Dilute the plasma with an equal part, or with twice the amount of normal saline (more in cases of jaundice); dilution diminishes the cloudiness of the plasma and gives a better matching.

Matching in the colorimeter.—The following adjustments of the scales and of the illumination must always be made each time before the colorimeter is used.

(*i*) Adjustment of the scales. Gently raise the cups until the bottoms are in contact with the plungers. The readings should be exactly at 0 on both sides. If they are not, they must be brought to 0 by adjustment.

(*ii*) Adjustment for uniform illumination. Half fill both the cups with the standard 1 : 10,000 potassium bichromate solution. Gently raise the cups until the bottoms touch the plungers—this will drive out any air bubbles that may be in the fluid.

Now slowly bring down the cups and set them at an equal depth, say 15 millimetres. Adjust the reflector in such a way that the same amount of light is reflected up through each cup.

Slight adjustment of the eyepiece may be necessary, if both halves of the field do not match exactly.

Taking a reading.—Keep the cup on the left side at a depth of 15 millimetres.

Take out the cup from the right side, throw out the potassium bichromate solution, wash the cup and plunger in water, dry well with soft linen. Put the diluted plasma into the cup and replace it on the right side of the colorimeter. Gently raise the cup until its bottom touches the plunger. Now move this cup containing the plasma up and down until a perfect match is obtained; take a reading. Move the cup up or down and again adjust it to match; take another reading.

Take the mean of three independent readings.

Calculation

$$\text{Icterus index} = \frac{\text{Reading of standard bichromate solution}}{\text{Reading of diluted plasma solution}} \times \text{dilution factor.}$$

Example.—One part of plasma is diluted with two parts of normal saline.
The cup containing the standard solution is at 15 mm.
The cup containing the unknown plasma matches the standard at 5 mm.

$$\text{Icterus index} = \frac{15}{5} \times 3 = 9.$$

B. *Matching in tubes.*—In the absence of a colorimeter, results sufficiently satisfactory for clinical purposes may be obtained by matching the plasma or serum diluted with normal saline against the standard 1 : 10,000 potassium bichromate solution.

Take two test-tubes of equal height and calibre. Into one put about 5 to 10 c.cm. of standard potassium bichromate solution. Into the other put 1 c.cm. of plasma and add measured amounts of normal saline from a graduated pipette until the colour matches the standard.

Calculation.—The dilution of the plasma required to match the standard potassium bichromate solution gives the icterus index.

Example.—To 1 c.cm. of plasma was added 7 c.cm. of normal saline to match the standard.
Dilution of the plasma is 1 in 8.
Therefore, icterus index = 8.

C. *Matching in a Lovibond comparator.*—If this comparator is available, it is an inexpensive matter to obtain an icterus index disc. The undiluted serum or plasma is placed in the right-hand tube and matched directly against the colour standards in the Lovibond comparator. When a correct matching is obtained, the icterus index is read from the right-hand corner of the comparator, as in the case of the van den Bergh test (*vide* figure 11).

The normal icterus index is 4 to 7.

Discussion.—The significance of the van den Bergh test in hæmatological investigation is that it gives a *broad* indication as to whether the anæmia is due to excessive blood destruction or deficient blood formation.

In the true hæmolytic anæmias the test always gives a high reading, 2·0 milligrammes or more, in iron-deficiency anæmias it is within normal limits, and in aplastic anæmias it may be completely 'negative' (in the normal individual there is usually a faint violet coloration of the serum).

In pernicious anæmia a reading well above the normal range is usual, for, though it is not a true hæmolytic anæmia, the deficiency of 'hæmopoietin' leads to the formation of abnormal cells which are very susceptible to destruction by the normal hæmolytic tissues.

In tropical macrocytic anæmias, in a large number of cases, especially in those with enlarged spleens, the test is much above the normal range and in a small number of cases the test is well within the normal range.

It is not, however, an absolute indication, either of excessive blood destruction or of deficient blood formation, as, for example, in an acute malarial attack, when there may be enough red cells destroyed to lower the red cell count a million or more cells per c.mm., the van den Bergh often remains within normal limits, for the liver cells are able to excrete this temporary excess of bilirubin in the blood, but if the red cell destruction is continued the bilirubinæmia will inevitably rise above the normal range and a 'positive' indirect van den Bergh reaction will result.

On the other hand, in liver dysfunction, not necessarily accompanied by anæmia, a positive van den Bergh may be found, for the liver cells are unable to utilize the products of normal hæmolysis.

9

The making and staining of blood films

Properly made and well-stained blood smears are essential for the white cell differential count, the enumeration of the nuclear lobes of neutrophil polymorphonuclears, as in the Arneth and Schilling counts, and also for the determination of the red cell size by the Price-Jones method.

For ordinary work, blood films are made on 3-by-1-inch glass slides and for special studies on 2-cm.-square coverslips: only new slides and coverslips should be used if this is possible. The slide and the coverslip must be thin, completely transparent, scrupulously clean, and free from grease and dust.

Cleaning glass slides and coverslips

Place the slides and coverslips in sulphuric-acid-bichromate mixture* and allow them to remain for 24 hours; this mixture should be kept in a large jar for the slides and in a small one for the coverslips. Decant off the cleaning mixture into another similar glass jar, which is then ready for use again. Transfer the slides or coverslips to a shallow enamelled tray or a big petri dish, and place this under a tap for 3 to 4 hours, stirring from time to time with a glass rod. Finally, wash with distilled water, dry with a piece of soft linen, and put them in a jar of absolute alcohol for 24 hours (methylated spirit may be used as a substitute). With a pair of forceps take out the slides or coverslips one by one, allow the excess of spirit to drain off, flame in a bunsen or spirit lamp, clean again with soft linen, and store in a dust-proof container—in a slide box for the slides and in a small petri dish for the coverslips. These are now ready for use for all ordinary work.

Polishing the slides

For special work, polish one side of a slide, cleaned as above, with jewellers' rouge for 2 to 3 minutes, clean with soft linen (selvyt cloth or silk is the best for this purpose), mark the polished side with a glass pencil, and store for future use in a dust-proof container.

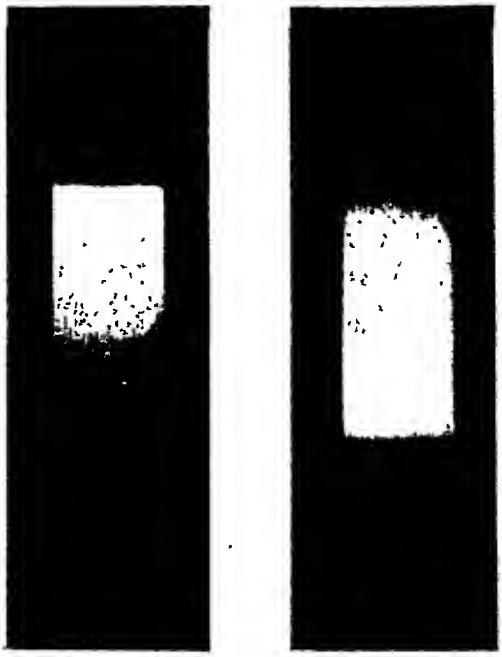
(A) Spreading the film in the routine use of venous blood

(*i*) *On slides.*—Put a few polished slides on a flat surface. With a syringe collect the blood from a vein (*vide supra*). Before putting the blood into the

---

* Concentrated sulphuric acid .. .. .. 100 c.cm.
Potassium bichromate (powdered) .. .. .. 100 gm.
Water .. .. to 1 litre.

oxalated flask and while the needle is still attached to the syringe, put a small drop of blood on to the middle of the slide, a little away from the end. Apply the thin edge of another slide, with the corners cut off so that the spreading edge is narrower than the slide, or better still of a spreader (hæmocytometer cover-slips make excellent spreaders on account of their smooth narrow edge), to the middle of the glass slide, slide it along until it makes contact with the drop of blood which will now spread along the edge of the spreader (slight lateral movement will accelerate this), then with the spreader at an angle of about 30° to 40° to the slide, push the spreader with the blood following it along the slide and raise the spreader abruptly just before the whole drop of blood has been used up. The film should be dried quickly by waving it in the air, or under an electric fan. An ideal film should occupy the middle third of the slide, should be of uniform thickness, should not have any tails, and when viewed under the microscope the red cells should just touch one another and there should not be any rouleaux formation anywhere on the slide.



(*a*) (*b*)

FIG. 12.

Figure 12*a* shows a satisfactory film but with 'tails', and figure 12*b* a good film with a straight 'leucocytic edge'. They were drawn from above downwards. Figure 13 shows the low-power view of a satisfactory leucocytic edge; the leucocytes are actually discrete though the photograph scarcely gives that impression.

FIG. 13.

The important points are a perfectly clean slide, a good spreader, and a drop of blood of the right size; this last can only be gauged by trial and error, but compared with the size of the drop required from a normal individual, a larger drop will be required from an anæmic patient and a smaller drop from one with polycythæmia or leukæmia.

(*ii*) *On coverslips.*—Place a few clean coverslips on a flat even surface. Proceeding as in (*i*) put a very small drop of blood on the centre of a coverslip; place another clean coverslip of the same size on the top of the drop of blood in such a way that the sides of the two coverslips are not opposed (*see* figure 14). If the two coverslips are clean, the blood will spread uniformly between them. Now draw apart the coverslips quickly but gently, and dry them quickly by waving them in the air or under an electric fan.

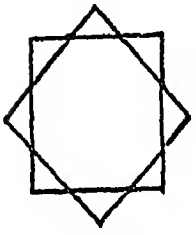


FIG. 14.

(B) FROM CAPILLARY BLOOD FROM THE FINGER OR EAR LOBE

Prick the finger or ear lobe fairly deeply with a sharp surgical needle or with a blood gun, so that the blood flows freely from the wound. Apply the surface of a clean slide or coverslip to a small drop of blood and proceed as in A (*i*) or (*ii*).

Comparing the two methods

The method of choice is in our opinion the slide smear. The advocates of the coverslip method claim that it is the only way to obtain an even distribution of cells and that consequently reliable differential counts can only be made from coverslips. On the other hand, it is much easier to make a good film on a slide, and, if the whole drop of blood on the slide is utilized in making the smear and this drop is a small one, examination of such a smear will give as reliable information as examining a coverslip smear, the preparation of which demands considerable dexterity.

Staining blood films

All blood smears should be stained within 24 hours. If the smears cannot be stained immediately they must be fixed with methyl alcohol and stored in a dust-proof slide box for staining at a later date. The unstained slides must never be left uncovered on the working table as blood is readily eaten by flies during the day and by cockroaches at night.

Romanowsky stains are used for all blood work. The most commonly used stains are those originated by Leishman, Wright, Jenner, and Giemsa. All these stains depend for their action on the compounds formed by the interaction of methylene blue and eosin, and the differences between the various stains are due to the proportion of the two stains. Excepting Giemsa's stain, the fluid stains are prepared by dissolving the dry powder in acetone-free pure methyl alcohol, so that a preliminary fixation with methyl alcohol is only required in the case of Giemsa's stain. Leishman's and Wright's stains are used in the strength of 0·15 per cent, and Jenner's stain in the strength of 0·5 per cent.

Preparing Leishman's, Wright's and Jenner's stains

Stains in powder or tablet forms and extra-pure acetone-free methyl alcohol for dissolving the stains should be obtained from some reliable firm. We have found the Gürr's* stains to be very satisfactory.

All the glassware used in preparing the stains and in storing them should be scrupulously clean, and free from any trace of water; they should be rinsed first with absolute alcohol and finally with a little methyl alcohol.

Take the requisite amount of stain in powder or tablet form in a small glass mortar. Measure out the requisite amount of methyl alcohol in a graduated glass cylinder. Pour out about 2 c.cm. of methyl alcohol on the stain and grind well to make it into a thin paste. Add in small quantities at a time about half the total amount of methyl alcohol, grinding all the time. Carefully decant the supernatant dissolved stain into a clean glass stoppered bottle. Add more methyl alcohol to the undissolved stain, grinding as before. Again decant the supernatant stain into the bottle, continue the process until all the methyl alcohol is used up. If this is properly done all the stain will go into solution and no residue will be left at the end. Incubate the bottle with the stain at 37°C. for 24 hours when it will be ready for use.

(A) Staining with Leishman's, Wright's or Jenner's stain

Reagents and apparatus required

(*i*) Prepared stain, preferably in a drop bottle.

(*ii*) Distilled water (pH 7·0), or buffer solution with pH of 6·4 and made with acid monopotassium phosphate and disodium phosphate†.

(*iii*) Fresh distilled water which is slightly acid in reaction.

(*iv*) Staining rack. This can be made with plasticine and a pair of glass rods of equal size and thickness, and may be placed at one end of a sink away from the water tap, or on a rectangular enamelled tray placed near a tap.

Glass beakers, cylinders, a pair of forceps, capillary pipettes, teats, etc.

Technique.—Put the slides on the staining rack taking care that the side with the blood film is upwards; also see that the two ends of the slides are in the same plane.

From a drop bottle, or with a pipette, pour on sufficient stain to cover the whole of the film; wait for one minute to allow for proper fixing; with a capillary pipette now add distilled water (pH 6·8 to 7·0) or the buffer solution—the same volume in the case of Leishman's and Wright's stains but double the volume in the case of Jenner's stains. With a capillary pipette or glass rod thoroughly mix the stain with the diluent to ensure a uniform mixture over the film.

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* George T. Gürr, 136, New Kings Road, London, S.W. 6, England.

† Monopotassium phosphate .. .. .. 0·03 gm.
Anhydrous disodium phosphate .. .. .. 2·56 gm.
Distilled water .. .. .. up to 1 litre.
Add 1 c.cm. of chloroform as preservative.

When the mixture is allowed to settle, a scum will form on the top, if the proportion of the stain and diluent has been correct. According to the depth of staining required, allow the diluted stain to act for 5 to 10 minutes, in the case of Leishman's and Wright's stains, and 3 to 5 minutes, in the case of Jenner's stain.

The diluted or undiluted stains on the slides must not be allowed to dry up at any stage of the staining. Drying is prevented by covering the staining rack with a wide bell-jar, or other improvised device*.

When the staining is complete, hold one end of the slide firmly with a pair of forceps, and place the stain-flooded slide under a running tap. This will wash off all the stain from the upper surface, while the bottom is cleaned by rubbing it well with the fingers of the left hand. The slide is now transferred to the beaker containing fresh distilled water and gently shaken to and fro until the colour of the smear becomes faintly pink. Now take it out of the beaker, wash again under the tap and allow it to dry. In order to dry it without allowing dust to adhere to the stained surface, the slide should be sloped against a vertical surface, e.g. a wall or the side of a box, with the film side inwards. (It is scarcely necessary to point out that heat should not be applied to a blood film at any stage in its preparation.)

When it is dry the slide is ready to be examined.

(B) Staining with Giemsa's stain

Note.—It is more difficult to prepare this stain and it is better to purchase it in solution. Giemsa's stain as prepared by Gürr is very satisfactory.

In staining with Giemsa's stain, preliminary fixing with methyl alcohol or some other fixing stain is absolutely necessary.

Preparing dilute solution

Take about 20 c.cm. of prepared distilled water (pH 7·0) or buffer solution in a clean transparent glass cylinder, add 20 drops of undiluted stain, or in other words as many drops of stain as there are cubic centimetres of water. Mix well by inverting the cylinder and see that the depth of colour of the mixture is such that, when held in front of the eyes, it allows a distant object to be seen through it.

(i) Place the slides to be stained on a staining rack, flood the slide with methyl alcohol and cover with a bell-jar, so that the methyl alcohol does not dry up on the slide. Allow the methyl alcohol to act for about 2 minutes, remove the bell-jar and thoroughly wash the slide with distilled water.

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* The precaution will seldom be necessary in humid climates, e.g. of Bengal and Assam, except in the hottest months, but will be imperative in drier provinces in India, and in other dry countries such as Iraq, Egypt, etc.

Now flood the slide with the diluted stain, cover with a bell-jar, and allow the stain to act overnight. Next morning wash and dry the slide as in A.

(*ii*) *Combined staining with Leishman's, Wright's or Jenner's and Giemsa's.*—This is done exactly in the same way as in A but substituting very dilute Giemsa's stain (1 drop in 2 c.cm.) as the diluent in place of distilled water or buffer solution.

(*iii*) *Combined staining with May-Grünwald* and Giemsa.*—Pour on undiluted May-Grünwald's stain just sufficient to cover the slide, place a bell-jar over the staining rack, allow the stain to act for 2 to 3 minutes, remove the bell-jar, and add an equal amount of neutral distilled water (pH 7). Allow the diluted stain to act for 5 to 10 minutes according to the depth of staining desired, wash thoroughly with neutral distilled water and then flood the slide with dilute Giemsa's stain to act for 15 to 30 minutes according to the depth of the staining desired, and wash and dry as in A.

Methods used in our laboratory

For all ordinary work Wright's staining method is used, and for special work, e.g. examining smears from the marrow, counting the nuclear lobes in Arneth and Schilling counts, combined May-Grünwald and Giemsa's staining are preferred. For, with the combined May-Grünwald and Giemsa's staining, all the component parts of both mature and immature red and white cells are very well shown.

10

White cell differential count

When making a white cell differential count, one should attempt to fulfil the following requirements :—

(*i*) The film should be uniformly spread and should be neither too thin nor too thick—the ideal film is one where the margins of the red cells when seen under the microscope just touch one another without overlapping (*vide* p. 77).

(*ii*) The film should occupy the middle two-thirds of the slide.

(*iii*) There should be no 'tails' at the end of the film.

(*iv*) The drop should be a small one so that the whole may be utilized in making the smear, as, if only a portion of a large drop is used, this portion may not contain a fair sample of the cells (e.g. the large cells tend to sink to the bottom of the drop).

* This can be purchased in solution and is available from G. T. Gürr (*v.s.*).

(*v*) The cells should be stained well and there should be no debris in between or on the cells.

A well-stained film shows—

red cells—an orange-buff colour, lymphocytes—with pale blue cytoplasm, and neutrophil granules—a dull lilac.

Number of cells to be counted

This will vary directly with the total white cell count, the higher the total count the larger is the number of the cells that should be counted to get a correct proportion of the different cells. The following rule may be taken as a guide, but need not be followed rigidly :—

Count 100 cells when the total white count is below 5,000 per c.mm.

Count 200 cells when the total white count is above 5,000 per c.mm. but below 10,000.

Count 300 cells when the total white count is above 10,000 per c.mm. but below 20,000.

Count 400 cells when the total white count is above 20,000 per c.mm.

The differential count should be done with an oil-immersion lens (one-twelfth) and a × 5 eyepiece with a × 10 eyepiece at hand. This magnification will enable one to see the details of the cells distinctly, but a higher magnification, given by the × 10 eyepiece, may sometimes seem desirable, e.g. in the case of doubt arising as to the true identity of a cell. In actual practice it will be found that one seldom changes the eyepiece.

The microscope must be fitted with a mechanical stage that allows easy movement in either the horizontal or the vertical plane. Northern daylight is preferable to artificial light, as it shows the true colour of the different cells. If artificial light has to be used a blue filter should be placed in front of the light itself or under the condenser of the microscope.

Procedure.—Place the stained blood film on the mechanical stage of the microscope and fix it securely; with the low-power (two-thirds objective) make a rapid survey of the different parts of the film, to find out if the requirements mentioned above are fulfilled. If not, it is recommended that another, properly made, film should be stained, for to attempt to make a differential count on an unsatisfactory blood film is laborious and irritating, and the result obtained is never accurate.

If the blood film is satisfactory, put a drop of cedar-wood oil on the smear, rotate the oil-immersion lens into position, open the diaphragm fully and raise the condenser to get the maximum amount of light. With the coarse adjustment, lower the oil-immersion lens just to touch the stained film through the cedar-wood oil; then, looking into the microscope, gently raise

the lens until the cells are seen, and finally manipulate the fine adjustment until the focus is accurate.

The distribution of the white cells in the different portions of the blood films depends on their size; the larger and heavier cells, e.g. neutrophils, eosinophils, and large mononuclears, are mostly found along the edges and at the tail end of a smear while the smaller and the lighter cells, e.g. the lymphocytes, tend to occupy the middle of the film.

Therefore, in order to get an absolutely correct differential count every cell in the blood smear on the slide, or on both the coverslips used in making the smear if the coverslip method is used, must be counted and classified, and the percentage calculated. This takes a long time, particularly if the smear is a large one; it is therefore advisable to make as small a smear as possible.

A very small smear can be made by taking a large drop, with the corner of a slide touching this drop, transferring a portion of it to another slide, and making a smear, but this must be done very quickly before the cells settle (*v.s.*). If the cells counted on a *whole* small smear are over a hundred, it is better to accept this than to supplement it by a counting *part* of another film.

Otherwise, a smear is made from a small (initial) drop and one of the methods suggested below is adopted. From neither of these methods will the error be very great.

(*i*) Start counting at a point A, near the proximal end of the smear and along one edge, moving the slide longitudinally, counting and classifying the cells all the time, up to B which is beyond the end of the film; move the slide the breadth of one or two fields to B′ and again move back along the whole length of the film to C, which is beyond the proximal end of the film; move across to C′ and then back again up and down the film. Continue to do this until about the middle of the film is reached, or until a sufficient number of cells has been counted, but always end the counting at the end of the film (*vide* figure 15).

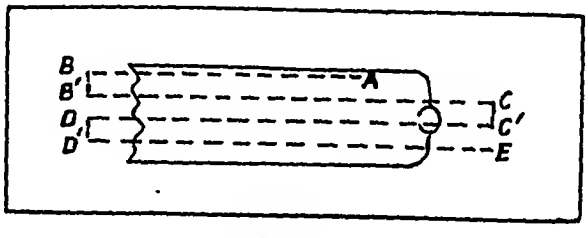


FIG. 15.

By this procedure you will have included one edge, and one or two rows of fields from the rest of the film. The majority of the heavy cells are drawn to the distal end and only a few to the sides, so that the discrepancy will not be great if, after counting one edge, you count a little more or a little less of the rest of the film. On the other hand, it is important to finish at the end, so that you will have covered an even proportion of body and tail of the film. For this reason an exact figure should not be aimed at, but the point of stopping, like the point of

starting, should be at one end of the film. The percentages of the different cells are then calculated.

Example.—Suppose that in making a differential white cell count of a blood film there are 141 neutrophils, 63 lymphocytes, 12 monocytes and 9 eosinophils, i.e. 225 cells altogether; the percentage of the different cells are calculated as follows:—

$$\text{Neutrophils} = \frac{141 \times 100}{225} = 62{\cdot}6, \text{ or } 62{\cdot}7 \text{ per cent.}$$

$$\text{Lymphocytes} = \frac{63 \times 100}{225} = 28{\cdot}0 \text{ ,,}$$

$$\text{Monocytes} = \frac{12 \times 100}{225} = 5{\cdot}3 \text{ or } 5{\cdot}3 \text{ ,,}$$

$$\text{Eosinophils} = \frac{9 \times 100}{225} = 4{\cdot}0 \text{ ,,}$$

(*ii*) *Four-field meander technique.*—Though this method is recommended in most textbooks we consider that it allows greater scope for individual variation in procedure, and we prefer the previous method; the method is as follows:—

From a point at the edge of the film move towards the centre of the film for about four 'fields', then, using the other mechanical stage adjustment, move about four fields towards the proximal end of the film, then back towards and beyond the edge, move a few fields towards the proximal end, and repeat the process until 50 cells have been counted and classified. This process should be repeated in four different places at the edge of the film, on either side and near each end of the film (*vide* figure 16).

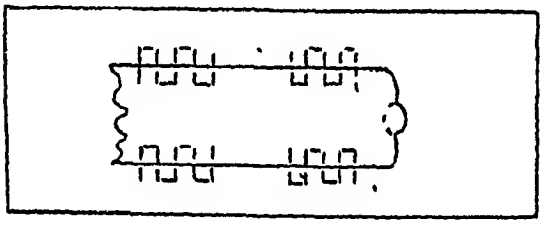


Fig. 16.

Recording the results

Except in cases of leukæmia and some rare pathological conditions, the types of white cell that are usually seen in the differential count of a peripheral blood film are neutrophils, lymphocytes, monocytes, eosinophils, and basophils, in the order of frequency in which they are encountered. As the slide is moved from one field of vision to another, the examiner must record both the total number and also the types of cell encountered.

This may be done in different ways:—

I. The usual method is to write down on a piece of paper the names of the cells in the order of frequency in which they usually occur in the peripheral blood and to put a vertical stroke against each name as that cell is seen in the field of vision, making the fifth stroke cross the other four, so that the strokes are in groups of five and easily counted (*vide* example 1). When all the cells in the film, or in the case of a large film, in the area decided upon, have been counted, they are totalled, and the percentage calculated.

Example 1.—

N — 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 III = 68

L — 𝍸 𝍸 𝍸 𝍸 𝍸 𝍸 II = 32

M — 𝍸 = 5

E — 𝍸 I = 6

B — I = 1

II. Another method is to memorize the numbers of the two main cell types until one of the other types is encountered, then to enter the count on your prepared list, and begin again (*vide* example 2).

Example 2.—

												Total.	Percentage.
Neutrophils	..	10	12	14	17	20	4	10	14	1	20	10	132 =	68·0
Lymphocytes	..	4	3	5	8	5	1	8	4	1	10	2	51 =	26·3
Monocytes	..	..	1	1	1	1	..	1	1	..	1	..	7 =	3·6
Eosinophils	..	1	..	..	..	..	1	..	..	1	..	..	3 =	1·5
Basophils	..	..	..	..	..	..	..	..	..	..	..	1	1 =	0·5
													194	

The numbers against each type are added and the percentages calculated.

III. A quicker method, which can be learnt with a little practice, is to remember the numbers of the different types of cell in the traditional sequence neutrophil, lymphocytes, monocytes, eosinophil and basophil until 25 cells have been counted ; the number of each type counted is entered against the respective name on a piece of paper and when the required number of cells have been counted, they are totalled and the percentages calculated (*vide* example 3).

Example 3.—

									Total.	Percentage.
Neutrophils ..	18	17	10	14	16	14	20	11	120 =	60·0
Lymphocytes	6	5	12	8	7	9	4	9	60 =	30·0
Monocytes ..	0	3	2	1	2	1	1	3	13 =	6·5
Eosinophils ..	1	0	1	1	0	1	0	2	6 =	3·0
Basophils ..	0	0	0	1	0	0	0	0	1 =	0·5
									200	

Method I is simple and fool-proof, but slow ; method II is also simple and saves the repeated interruptions of method I ; method III is very popular, but tends to

make one stop at a round number and thereby simplify the calculation, which is a procedure not in the best interests of accurate countings.

In methods I and II, theoretically one has to stop periodically and add up all the figures to see if sufficient cells have been counted, but this can be obviated by the use of a mechanical counter, e.g. the Veeder-Root hand tally. The 'counter' fits nicely into the hollow of the palm and is conveniently kept in the left hand. The knob is pressed with the thumb each time that a cell is seen and the numbers are at once recorded on the dial. The total number of cells counted at any time during the process of the differential count can be seen by simply looking at the dial of the counter.

Expressing the results

It is customary to express the result in the white cell differential count as a percentage of the various cells encountered, without any reference to the total white cell count. This does not always convey the full significance of the observation, and both percentage and absolute figures should be given.

The absolute figures are readily obtained by striking out the last two zeroes of the total leucocyte count, or moving the decimal point back two spaces, and multiplying each percentage by this figure, as in the example given below:—

Example.—(A fairly normal count).

Total white cell count—7,500 per c.mm.

Differential white cell count—

	Per cent.	Total per c.mm.
Neutrophils	68·0	5,100
Lymphocytes	24·0	1,800
Monocytes	5·0	375
Eosinophils	2·5	187
Basophils	0·5	37

Now, if the patient were to take some toxic drug which reduced the granulocytes to a low figure, say to 540 per c.mm., but did not affect the other leucocytes, the total count would now be 2,700 per c.mm. and the percentages—

	Per cent.	Total per c.mm.
Neutrophils	19	513
Lymphocytes	67	1,809
Monocytes	13	351
Eosinophils	1	27
Basophils	0	0

In this count the attention is attracted by the high lymphocyte and monocyte percentages, whereas actually these cell elements are normal and all that has happened is a decrease in neutrophils.

Again, in asthma there may be a sudden high rise in eosinophils which reduces considerably the percentage of neutrophils, even in cases where there has been an actual increase in this element, so that again a wrong impression is made.

Therefore, a differential count should always be given in absolute figures as well as in percentages.

Normal white cell counts.—The normal leucocyte count in an adult is usually given as 7,000 to 9,000 per c.mm. Some of our findings in Indian populations are given in Table IX.

TABLE IX

Leucocyte counts in 'normal' individuals

Subjects		Residence	Total leucocyte count		Monocytes		Eosinophils	
Number	Sex		Mean	S. D.	Mean	S. D.	Mean	S. D.
50	Males ..	Calcutta	6,542	± 1,214	7·00	± 3·91	6·90	± 5·19
128	Females	„	7,162	± 1,705	..		5·16	± 4·21
24	Males ..	Assam	8,160	± 2,650	9·44	± 4·32	13·76	± 8·8
17	Females	„	8,768		8·35		13·74	
25	Males ..	Cachar	7,111	± 1,759	..		..	
25	Females	„	6,456	± 1,755	..		..	

It will be noted that the monocyte counts are all above the usual normal figure, but especially those in Assam, where malaria is very prevalent. The eosinophil figures are also high compared with European and American standards and they are exceptionally high in the Assam population, where the hookworm infection rate is nearly 100 per cent.

From records of 114 normal females in Calcutta in which the full differential counts are available, we have calculated the following means :—

	Percentage.	Per c.mm.
Neutrophils	62·6	4,507
Lymphocytes	28·9	1,937
Monocytes	5·5	396
Eosinophils	4·8	346
Basophils	0·2	14

11

Cell identification

The first essential for accurate identification of the cells of the blood or marrow is a satisfactory and properly stained blood film. A well-stained film should show no precipitate on or between the cells, the red cells should stain an orange buff, the neutrophil granules a dull shade of lilac, and in the monocyte the so-called 'azurophil granules' should just be visible—with any of the Romanowsky stains.

The following system is recommended for the identification of different cells.

Granules.—First, ascertain whether the cytoplasm of the cells contains any granules, or not. If there are granules, decide whether the granules are neutrophilic, eosinophilic, or basophilic.

Neutrophil granules are small, uniform in size, uncountably numerous, and stain a dull shade of lilac.

Eosinophil granules are large, round, uniform in size, and stain orange-red with pale centres. They are not so numerous as neutrophil granules—occasionally eosinophil granules take on a bluish stain, even in a well-stained specimen, while in a badly stained one all may be bluish.

Basophil granules vary considerably in size in the same cell, they stain a dark blue-violet colour, which is entirely different from the colour of the nucleus; they are always coarser and fewer in number than the neutrophil or eosinophil granules. The granules are always found superimposed on the nucleus, as well as in the cytoplasm.

Besides these specific granules, the presence of which characterizes the cells of the granular series, some cells of the non-granular series, e.g. monocytes and lymphocytes, may show some stained particles in the cytoplasm of the cells which have been called 'azurophil granules'. These azurophil particles are of the same colour as the nucleus of the cell in which they occur; but may be paler, brighter, or darker. They vary in size, in depth of staining and in number. In the lymphocytes the particles are few in number, they are large, coarse, and darkly stained, and occur in small groups in the cytoplasm, while the particles in the monocytes are more numerous, paler and finer, and are more evenly distributed in the cytoplasm.

Having noted whether granules are present or not, and if present the nature of the granules, employ the following tables for the further identification of the cells:—

Table X gives the cells of the non-granular series, and Table XI those of the granular series.

The cells of the granular series all originate from the primary differentiated cell of that series, the myeloblast. They are arranged in the table in order of maturation.

TABLE X

Identification of cells without specific granules

Nucleoli	Shape of nucleus	Chromatin structure	Size of nucleus in relation to cell	Cytoplasm	Azurophil particles	Name of cell	Identification mark
Present	Round, oval, or irregular.	Fine	More than three-quarters	Clear light blue	None	Mycloblast	I
		Finer than I	" " "	Clear deeper blue than I		Lymphoblast	II
		Relatively coarse	" " two-thirds	Light blue, opaque		Monoblast	III
	Ditto	Fine, stippled	" " "	Dark grey-blue, ground-glass with clear halo around the nucleus.		Megaloblast	IV
Present or absent.	Round, oval, or irregular	Coarse	More than half	Clear light blue	May be present	Large lymphocyte	IIa
	Kidney or irregular	Coarser than III	" " "	Light blue, opaque		Pre-monocyte	IIIa
	Generally round or oval; may be clover leaf.	Coarse; in clumps	More than half in larger cells but almost fills the cell in the smaller ones.	Clear transparent blue; may appear only as a thin rim in the smaller cells.	May be present; few; unevenly distributed.	Lymphocyte	IIb
Absent	Horse-shoe or irregular	Coarse clumps or strands.	Half or more	Light, faded blue, opaque	Usually present, very fine, numerous, scattered.	Monocytes	IIIb
	Round or oval	Coarse	More than two-thirds	Ground-glass greyish-blue, with a little hæmoglobin in some cases.	None	Erythroblast	IVa
		Very coarse	Variable, usually less than two-thirds.	Ditto with more hæmoglobin.		Macroblast	IVb
	Round	Pycnotic (nucleus almost black).	Variable, often less than half.	Grey, reddish-grey, or pink as in the red cells.		Normoblast	IVc
	Round or oval	Very coarse, cart-wheel appearance; eccentric.	Less than half	Deep greyish-blue ground-glass.	None	Plasma cell	V
	Irregular 'multi-lobed'	Coarse	Variable	Light blue obscured by azure particles.	Present, fine, numerous.	Megakaryocyte*	VI

* Mature platelets (*VIa*) which develop from the megakaryocyte are small (2 to 3μ) ill-defined structures, not clearly differentiated into nucleus and cytoplasm.

The mature neutrophil, eosinophil and basophil have their specific precursors in the earlier cells of the series and these can be distinguished by the size and colour of their granules (*v.s.*); there are, thus, neutrophil, eosinophil and basophil of each developmental stage, pre-myelocytes, myelocytes, meta-myelocytes and staff cells.

Table XII is a supplementary table to aid in the identification of primary differentiated cells which are already shown in Table X.

The points of difference in the morphology of the different primary cells are very fine and at times it is almost impossible to distinguish one from the other. In cases of leukæmia or anæmia the identification of an individual cell has often to be made, not on the cell alone but on the characteristics displayed by other associated cells; in other words, the cell is judged 'on the company it keeps'.

Table XIII is also supplementary. It gives the nucleated cells of the red cell series in order of maturation.

The development of blood cells

We do not propose to discuss the origin of the cells found in the blood stream, for it is a controversial field in which 'unitarians', 'dualists', and 'trialists', of the monophyletic and polyphyletic schools argue with one another to the confusion of the practical hæmatologist. We are only concerned with the cells already differentiated, the immediate precursors of the normal and abnormal cells of the peripheral circulation.

It is, however, impossible to avoid controversy altogether, as this is intimately associated with nomenclature which is definitely our domain, for at present much confusion in the literature is due to different writers adopting different names for the same cell, and the same name for different cells.

We have followed the Sabin school in using the word 'megaloblast' for the earliest differentiated precursor of both normal and abnormal red cells, and have used the expression 'Ehrlich's megaloblast' for the hæmoglobinized megaloblasts that are encountered in Addison's pernicious anæmia and rarely in other macrocytic anæmias. Some writers refer to 'our' normal megaloblast as a basophil erythroblast, an early primary erythroblast, a prenormoblast, or even as a hæmocytoblast (which name most writers reserve for the common stem cell).

With reference to the other cells, we have taken the view that the granulocytes, lymphocytes, monocytes and platelets have each a separate differentiated precursor cell, and following the usual practice we have called these myeloblast, lymphoblast, monoblast, and megakaryocyte, respectively.

TABLE XI

Identification of cells containing granules

Nucleoli	Nucleus	Granules	Cytoplasm	Cell	Mark	Remarks
Present ..	Round or oval; relatively fine chromatin structure.	Very few; fine, scattered.	Pale blue. Relatively small amount.	Pro-myelocyte	*Ia*	This, as well as the other granular cells, may be neutrophil, eosinophil or basophil. Basophil pro-myelocytes are rarely identified.
Generally absent.	Round or oval; coarse chromatin structure; may not be very well stained.	Very prominent; coarse.	Very pale blue; variable in amount, but usually more than in *Ia*; not well seen, as it is covered by granules.	Myelocyte	*Ib*	In the eosinophil and basophil myelocytes, the cytoplasm is almost entirely covered by granules, and is rarely visible. In the basophil the nucleus also is blurred by granules.
Absent	Bean or kidney-shaped; coarse chromatin structure.	Granules less prominent than corresponding granules in *Ib*; numerous.	Large in amount. Very pale blue obscured by granules.	Meta-myelocyte or young forms.	*Ic*	The basophil meta-myelocyte and staff cells are very difficult to identify.
	Curved rod; coarse chromatin structure.	Finer and more numerous than corresponding granules in *Ic*.	Large amount. Very pale blue.	Staff (or band)	*Id*	
	Lobed or segmented, 2 to 5 or more lobes.	Very fine and numerous; light lilac colour.	Large amount. Very light greyish-blue.	Neutrophil polymorphonuclear granulocyte.	*Ie (i)*	
	Usually bilobed; rarely may have more lobes.	Finer than eosinophilic precursor *Id (ii)*, but coarser than *Ie (i)*.	Very pale blue; scarcely seen.	Eosinophil granulocyte.	*Ie (ii)*	
	Segmentation of the lobes is difficult to make out, but never more than two lobes.	Very coarse; dark violet blue, almost black, often obscuring nucleus.	Very pale blue; scarcely seen.	Basophil granulocyte.	*Ie (iii)*	

TABLE XII.

Identification of the earliest differentiated cells

Name of cell	Nucleoli	Nucleus	Chromatin	Nuclear membrane	Cytoplasm	Auer's bodies
I. Myeloblast ..	Few, not very distinct.	Round or oval; usually central.	Fine reticulation	Not distinct and no condensation of chromatin at edges of nucleus.	Clear light transparent blue.	May be seen occasionally.
II. Lymphoblast ..	Many, very distinct.	Round or oval and central.	Finer reticulation	Distinct with condensation of chromatin at the edges.	Clear deeper blue.	Not seen.
III. Monoblast ..	One or two often clean cut.	Round or oval; often folded.	Relatively coarse	Not distinct and no condensation of chromatin at edges.	Light blue, not so transparent.	Frequently seen.
IV. Megaloblast ..	Few, appear as irregular gaps.	Round or oval	Fine, stippled	Not distinct and no condensation of chromatin at edges.	Grey blue ground-glass, with halo round nucleus.	None.

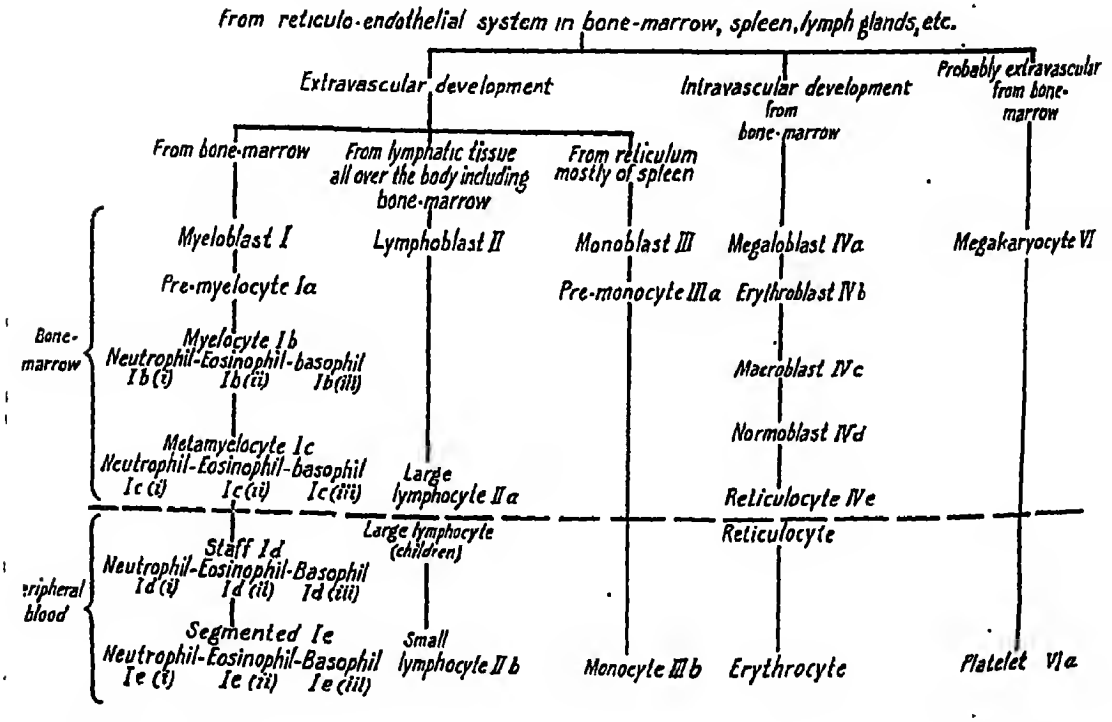
TABLE XIII

Identification of the different nucleated cells of red cell series

Cell	Size	Cell outline	Cytoplasm	Nucleoli	Nucleus	Chromatin
IV. Megaloblast ..	16–21μ	Irregular	(i) Dark blue grey (basophilic). (ii) Polychromatic or cosinophilic (Ehrlich's megaloblast).	Present, few, appear as irregular gaps.	Large; occupies more than two-thirds of the cell; sometimes eccentric.	Fine, stippled, stains lightly.
IV*a*. Erythroblast ..	12–18μ	Regular	Basophilic, polychromatic or cosinophilic.	Absent	Central and smaller than that of (IV); occupies over two-thirds of cell.	Coarse, less reticular, deeply-staining.
IV*b*. Macroblast ..	7–14μ	Regular	Basophilic, polychromatic or cosinophilic.	Absent	Usually central and occupies more than half.	Very coarse, reticular, deeply-staining.
IV*c*. Normoblast ..	5–10μ	Regular	Polychromatic or cosinophilic.	Absent	Sometimes eccentric; may be lobed or clover leaf. Relative size variable.	Pycnotic, appears as drop of ink.

Red cells.—Normally, the red cells originate intravascularly from the endothelial cells of the sinusoidal spaces and capillaries of the bone marrow. The first differentiated cell is the megaloblast. As development proceeds, the megaloblast passes through the stages of erythroblast, macroblast, normoblast and reticulocyte to develop into the mature erythrocyte (*vide* schema). In the process of maturation the nucleus loses its nucleoli very early, so that these are not seen in the erythroblast; the nucleus also loses its reticular structure and becomes gradually coarser and coarser as development proceeds, until in the normoblast it becomes completely pycnotic; after this the nucleus is lost (by fragmentation or extrusion) and the erythrocyte becomes fully mature. The reticulocyte is a

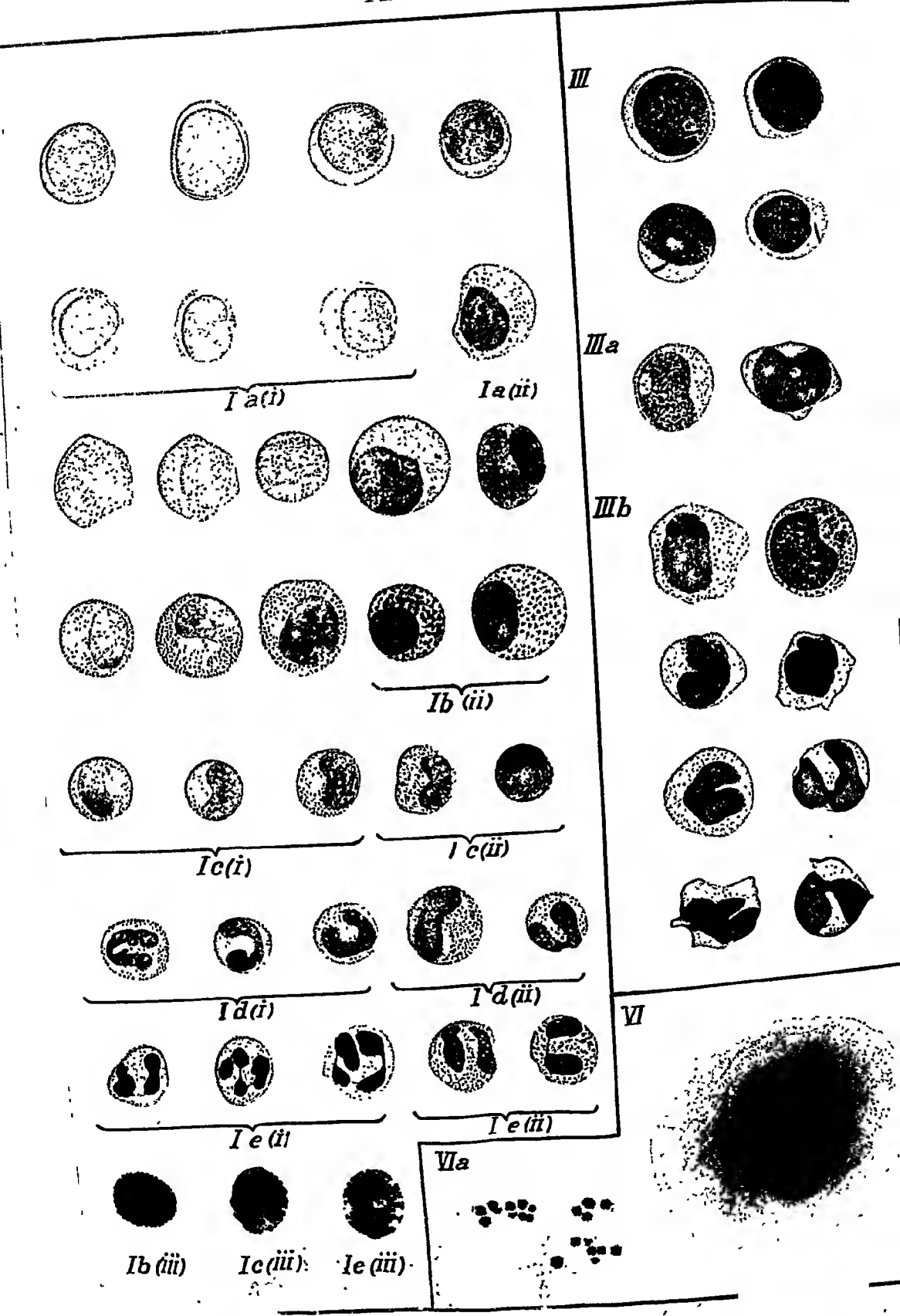
SCHEMA SHOWING ORIGIN OF BLOOD CELLS.



stage between the normoblast and the fully-mature red cell, but the reticulations are not seen in a Romanowsky-stained film.

Granulocytes.—The granular cells develop extravascularly from the reticulo-endothelial cells of the bone marrow. The earliest differentiated cell is the myeloblast.

The first evidence of maturation of the myeloblast is the appearance of granules in the cytoplasm. At first the granules are very fine and few, as in the pre-myelocytes, but gradually they become more numerous and coarser and are coarsest at the myelocytic stage, after which the granules again become finer and less numerous as development proceeds. In the fully-developed neutrophil polymorphonuclear cells the granules are very fine and numerous. The cells can


III
IIIa
IIIb
I a(i)
I a(ii)
I b (ii)
I c(i)
I c(ii)
I d(i)
I d(ii)
I e (i)
I e (ii)
VI
VIa
Ib (iii)
Ic (iii)
Ie (iii)

Explanation of Plate I

I. *Myeloblasts.*

I*a*. *Pre-myelocytes* showing azure particles: the fourth cell, an eosinophil precursor, is slightly more mature than the rest.

I*b*. *Myelocytes;* the cells in the upper are less mature than those in the lower row: the first three in each row are neutrophils and the last two eosinophils.

I*c*. *Meta-myelocytes;* the last two I*c*(*ii*) are eosinophils (the last cell in the row has not been reproduced well and has lost the red coloration that was apparent in the original drawing).

I*d*. *Staff* or *band* forms, the last two being eosinophils I*d*(*ii*). (The colour of the last two is too red; the purple colour of the nucleus is sometimes obscured by the eosinophil granules, but not to the extent indicated here.)

I*e*. *Mature granulocytes,* the first three being neutrophils and the last two eosinophils.

I*b*(*iii*), I*c*(*iii*) and I*e*(*iii*). *Basophils,* myelocyte, meta-myelocyte and mature. The shape of the nucleus is the only feature on which the immature forms are identified. In the original drawing the shapes of the nuclei were just discernible, but in the reproduction they are obscured.

III. *Monoblasts,* the lower two showing Auer's bodies.

III*a*. *Premonocytes.*

III*b*. *Monocytes;* the second cell in the top row might be classed as a premonocyte, but the azure particles are well developed. (There should be more blue colour in the cytoplasm of these cells.)

VI. Megakaryocyte; these cells are often proportionately much bigger than the one shown.

VI*a*. Platelets.

Magnification about × 1,000.

Staining—May-Grünwald and Giemsa.

Explanation of Plate II

IV. *Megaloblasts* (in the fourth cell the nucleus is much too red).

IV*a*. *Erythroblasts;* in the fourth cell a considerable amount of hæmoglobin has developed.

IV*b*. *Macroblasts;* the second and third are well hæmoglobinized; the last two are small varieties.

IV*c*. *Normoblasts;* in the second cell the nucleus is dividing and about to be extruded.

IV*d*. *Reticulocytes.*

IV*e*. *Erythrocytes:* mature red cells.

(*i*) Red cell showing Howell-Jolly bodies.

(*ii*) Red cell showing Cabot's rings.

(*iii*) Misshapen red cell.

(*iv*) Red cell with basophilic stippling.

II. *Lymphoblasts.*

II*a*. *Large lymphocytes* with azure particles.

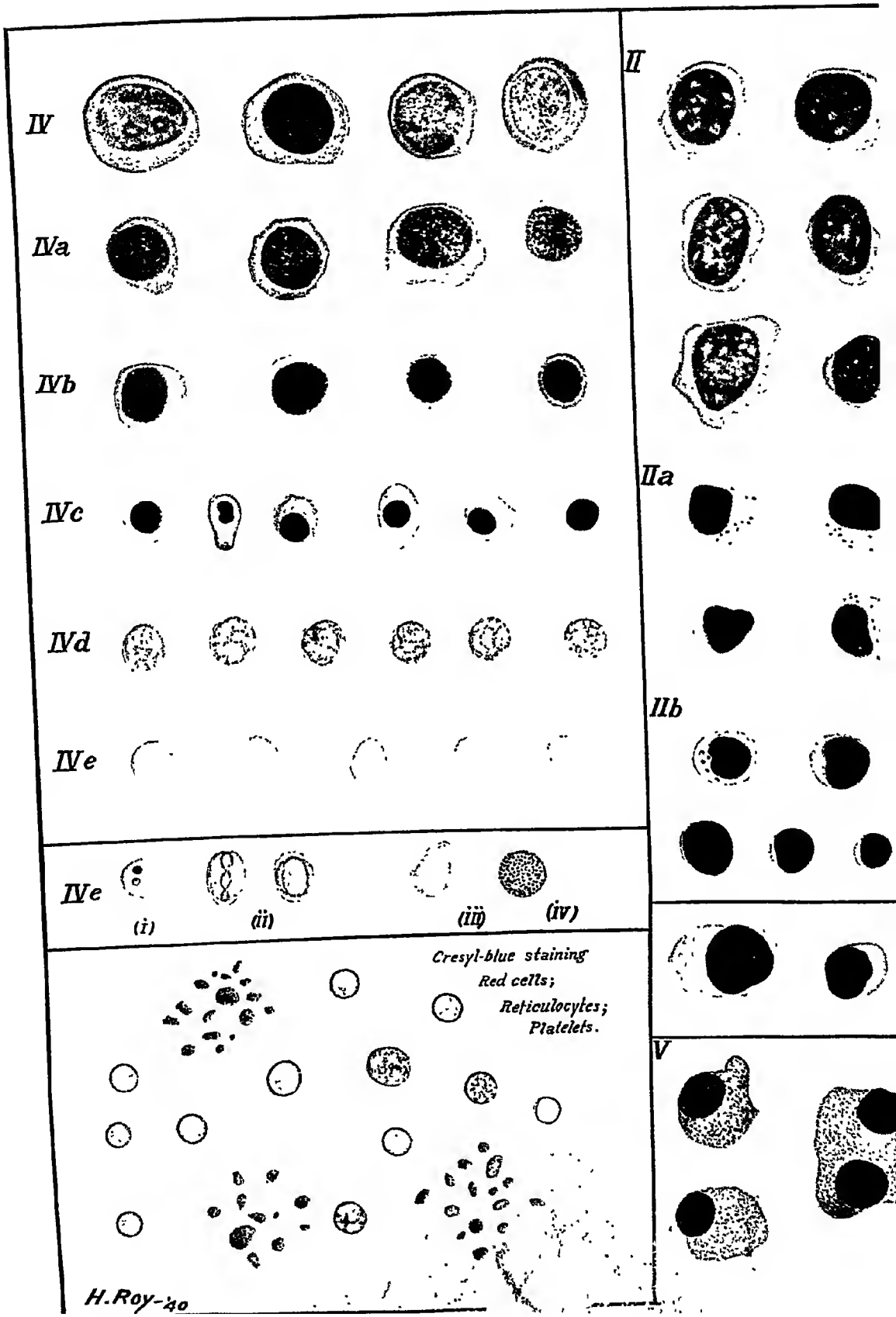
II*b*. Mature (small) *lymphocytes:* the first with azure particles. Below the lymphocyte series are two *Turk's cells;* the characteristic cart-wheel structure of the nucleus is not very clear.

V. *Plasma cells.*

Cresyl-blue supra-vital staining (*see* p. 58).
Erythrocytes: reticulocytes: platelets.

Magnification about × 1,000.

Staining—May-Grünwald and Giemsa, except figures in row IV*d* which are stained first with cresyl-blue and then Wright's stain [*vide* p. 57, method A(*b*), *i*], and the block in the left lower corner in which all the cells are supra-vitally stained with cresyl-blue (*vide* p. 58, method B*ii*).


IV
IVa
IVb
IVc
IVd
IVe
IVe
(i)
(ii)
(iii)
(iv)
Cresyl-blue staining
Red cells;
Reticulocytes;
Platelets.
H.Roy-'40
II
IIa
IIb
V

be differentiated as neutrophil, eosinophil and basophil according to the colour and size of their respective granules, with difficulty at the pre-myelocyte stage but quite easily from the myelocyte stage onwards.

In the process of development the nucleus loses its nucleoli; these are ill-defined in the pre-myelocyte and are almost always absent from the myelocyte stage onwards. The chromatin structure of the nucleus which is finely reticular in the myeloblast becomes coarser and more condensed as development proceeds, and is very dense at the later stages; this condensation makes the nucleus smaller. The shape of the nucleus too changes from round or oval at the earlier myeloblast, pre-myelocyte, and myelocyte stages to kidney or bean shape at the meta-myelocyte stage, and then it becomes elongated in the staff, and finally lobulated in the segmented forms.

The cytoplasm which is blue and scanty in the myeloblast loses its colour gradually and becomes more abundant as development proceeds—in the later stages the cytoplasm is hardly visible as it is covered by the granules.

Monocytes.—The monocytes originate from the reticulum all over the body but particularly from that in the spleen. The first differentiated cell is the monoblast; it proceeds through the stage of the pre-monocyte to the stage of fully-developed monocyte. As development proceeds, the nucleus loses its nucleoli and the fine reticular structure becomes coarser, and very fine and numerous azure particles appear in the cytoplasm of a fully-developed monocyte. The nucleus which is round or oval in the monoblast, becomes slightly indented in the pre-monocyte, and may take on various shapes in the mature monocytes. (We have made no attempt to differentiate between monocytes and histiocytes; this can only be done by a supra-vital staining process which we are not describing here. Some of the large mononuclear cells in the peripheral blood are undoubtedly histiocytes, whose origin is probably different from that of the monocytes.)

Lymphocytes

The lymphocytes originate mainly from the lymphatic tissue of the lymph glands all over the body and to a small extent from the lymphoid tissue in the marrow. The first differentiated cell is the lymphoblast which proceeds through the stage of large lymphocyte to the fully-developed (small) lymphocyte.

In the process of development the nucleus loses the nucleoli, the fine chromatin structure becomes coarser, and finally in the mature cells it is pycnotic. The cytoplasm which is definitely blue in the lymphoblast becomes lighter in colour as development proceeds, but is always transparent. In some of the cells a few coarse irregularly scattered azure particles may be seen.

12

Technique of sternal puncture

Bone puncture as an aid to accurate diagnosis of blood diseases and to the study of their ætiology has only come into general use during the last few years;

the main reason for this was that the methods used for obtaining material from the bones were comparatively difficult, and usually painful, unless an anæsthetic was given, before the Salah sternal-puncture needle was introduced. This handy and inexpensive instrument is now used widely not only in hæmatological work but in the diagnosis of kala-azar and other protozoal and bacterial diseases. We have used this needle for about four years and have adopted sternal puncture as a routine procedure in all cases of anæmia.

The senior writer described this technique in a paper written in co-operation with Dr. P. C. Sen Gupta* (Napier and Sen Gupta, 1938); since then, as a result of further experience, certain modifications in technique have been introduced and these are incorporated in this description, but the technique described is materially the same as that given in the above-mentioned paper.

Apparatus required

(*i*) The sternal-puncture needle.
(*ii*) Two Record syringes, 2 c.cm.
(*iii*) A pair of scissors and a shaving set.
(*iv*) Two per cent solution of novocaine or any of its substitutes. (Pitumerit 4 per cent solution produces good anæsthesia.)
(*v*) Absolute alcohol and ether.
(*vi*) Collodion or tincture of benzoin.
(*vii*) Cotton-wool, etc.

The needle is dry-sterilized in a hot-air sterilizer in a test-tube. The syringe is sterilized by boiling and then dried thoroughly by first driving out water with alcohol and later alcohol with ether, and finally by drawing in and expelling hot air (through the flame of a gas burner or spirit lamp).

The sternal-puncture needle

The Salah needle used for sternal puncture is shown (figure 17). It is made of rustless steel and the bore is about the same as that of a lumbar-puncture needle. The guard C on the needle can be moved so as to adjust the depth of the puncture. Usually the guard has to be fixed at a distance of 1 to 1·5 cm. from the tip, in order that the marrow may be reached. This distance will vary with the thickness of the skin and subcutaneous tissue of the thoracic wall; in fat individuals up to 2 cm. may be required and in very emaciated ones less than 1 cm. It may be found

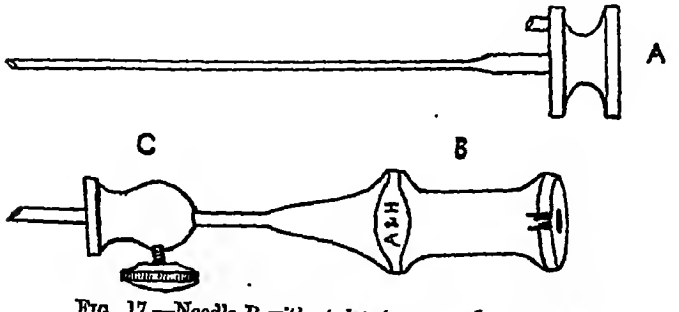


FIG. 17.—Needle B with stylet A removed: movable guard C (actual size).

* With whose kind permission the figures from this paper have been reproduced.

The apparatus is held with the knob of the stylet in the palm of the hand and the needle itself between the thumb and index finger, the latter on the guard C of the needle. Pressure is applied and the skin and subcutaneous tissues are pierced; a rotary movement will then facilitate puncture of the outer plate of the sternum (figure 18). As the external plate of the sternum is pierced and the marrow cavity is entered, there is a sensation of loss of resistance, just as is felt on entering the spinal canal during lumbar puncture. The stylet is now taken out, the 2-c.cm. Record syringe is attached to the end of the needle, and the marrow blood is aspirated. When the fluid is aspirated the patient feels a dragging pain (figure 19) which is

FIG. 19.—The stylet has been removed and sinusoidal blood is being drawn into a Record syringe, usually the only painful part of the operation.

a guide as to whether the needle is in the marrow cavity or not. About 0·5 c.cm. of marrow (sinusoidal) blood is removed and the syringe and the sternal-puncture needle are withdrawn; digital pressure is applied over the puncture for a minute or two and the puncture is sealed with collodion. The needle is detached from the syringe and the latter is inverted several times so as to mix the contents thoroughly; then small drops are placed on clean slides and smears are made (*vide* p. 77). The rest of the fluid is put into an oxalate tube.

Only very rarely will one fail to obtain blood. The commonest error is to fail to allow a sufficient length of needle. In this case the guard must be adjusted

slightly, the stylet replaced, and the needle pushed in a little deeper. Occasionally, the needle goes too deeply and has to be withdrawn slightly before blood will come. Our only complete failure was in a case of leukæmia, and in none of our cases of leukæmia has the blood come freely.

Not more than 0·5 c.cm. of blood is aspirated, because, if more is drawn, there is a probability that the negative pressure in the marrow cavity will draw blood from the vessels in the locality, and thus dilute the sinusoidal blood. It is probably impossible entirely to prevent this occurring; therefore a constant amount of blood is drawn to obviate gross differences in the degree of dilution in the samples taken from different persons. The syringe is inverted several times in order that the contents may be mixed thoroughly. This is necessary because the fluid that comes out at the beginning is not the same as regards cellular content as the fluid that comes out towards the end of the aspiration*.

Examination of material

(1) Examination of the stained smears:—

The sternal puncture smears are best stained with combined May-Grünwald and Giemsa staining; this shows the nuclear details very clearly. Failing this, satisfactory results for ordinary work may be obtained by staining with Leishman's or Wright's stain (*vide* p. 81).

An accurate differential count of the different nucleated cells is made by counting 500 or more nucleated cells from different parts of a well-stained smear. The criteria on which the different cells are identified are given above.

(2) The oxalated specimen is examined for:—

(*a*) enumeration of total nucleated cells (*vide* p. 51),

(*b*) estimation of hæmoglobin (*vide* p. 40),

(*c*) enumeration of red cell (*vide* p. 49), and

(*d*) estimation of reticulocyte percentage (*vide* p. 58).

Discussion

The material which is obtained by this procedure is neither peripheral blood (obviously) nor bone marrow, but is blood from both the patent and the closed sinusoidal spaces in the hæmopoietic tissue of the bone marrow, in which are mixed a few cells detached from the walls of these sinuses by the intruding needle, or by the act of aspiration. The extent to which the detached tissue cells are added to the sinusoidal blood probably varies with each puncture and constitutes the weakness of the procedure from the point of view of obtaining a true and unvarying picture of bone marrow hæmopoietic activity. However, the extreme variations in a single subject that have been reported by some workers and the differences in

* Vide *Ann. Rep., Calcutta School of Trop. Med. for* 1938.

the normals given by different workers can probably to some extent be accounted for by variations or defects in the technique employed, e.g. by the failure to remove a small and constant amount of blood, by failure to mix the blood before making smears, or by making counts from one part of the blood film only.

Zanaty (1937) has pointed out that wide variations may exist in the total nucleated cell counts and that from these counts little idea of the activity of the bone marrow can be obtained, but from our experience of sternal puncture we believe that there is generally a distinct correlation between hæmopoietic activity and the total nucleated cell count, though this may on occasions be misleading.

Both the hæmoglobin and red cell count are slightly lower and reticulocyte count slightly higher in the sinusoidal blood than in the venous blood, but these estimations are of little practical value.

Normal standards

It is useful to know what proportions of the various cells one may expect to find in a sternal puncture. The data given by various writers are difficult to correlate, because they have adopted different methods of classification and different nomenclatures, and we prefer to quote only our own findings.

The data given in Tables XIV and XV are from two sources:—

(*a*) First series: 10 normal individuals, males; 2 c.cm. of fluid taken.

(*b*) Second series: 53 subjects infected with filariasis; 0·5 c.cm. of fluid taken.

We have quoted the latter series because we believe that they represent the findings in normal individuals very closely, and because it is not easy to persuade 50 perfectly healthy individuals to undergo a sternal puncture. One might expect them to differ from the normal in the matter of the eosinophil cells, but in actual fact the percentage is lower than in the first normal series. The material differences that will be noted between the two series are almost certainly due mainly to the fact that in the second series we took a smaller amount of fluid and that there was consequently less dilution by systemic blood.

The main discrepancies are the higher total nucleated cell count in the second series—higher than the 'normal' figure usually quoted, a lower mycloid/nucleated-red-cell ratio—also lower than the usual normal figure, a higher lymphocyte percentage, a higher staff neutrophil percentage with correspondingly lower percentages of both young forms and segmented neutrophils—which may be due to the personal factor, and a higher maximum figure for most of the cells—which may be due to the larger number of subjects involved in the second series: the 63·2 per cent for nucleated red cells can scarcely be classed as 'normal'.

For the findings of other workers reference should be made to Zanaty (*loc. cit.*) and Scott (1939).

TABLE XIV

Sternal puncture: normal data

	First series		Second series	
	Mean	Standard deviation	Mean	Standard deviation
Hæmoglobin in grammes per 100 c.cm. ..	13·43	± 0·93	..	..
Red cells per c.cm. in millions	4·99	± 0·40	..	..
Reticulocytes—percentage of red cells ..	0·75	± 0·30	..	..
Total nucleated cells per c.cm.	53,500	±26,500	111,078	± 64,832
Nucleated red cells—percentage of total nucleated cells.	25·75	± 4·07	27·606	± 11·746
Leucocytes—percentage of total nucleated cells	74·25	± 4·07	..	..
Granulocytes—percentage of total nucleated cells	64·40	± 5·10	54·529	± 11·421
Non-granular leucocytes—percentage of total leucocytes.	9·85	± 2·43	17·889	± 6·796
Myeloid/nucleated-red-cell ratio	2·50	..	1·975	..

Many writers claim that megaloblasts are not present in the normal sternal puncture material. This is a matter of nomenclature. The cell we have described as a megaloblast (*v.s.*) is seen in the normal marrow.

We seldom identify cells as lymphoblasts or monoblasts except in cases of leukæmia but myeloblasts are occasionally found and appear in most of our counts, forming 0·1 to 0·2 per cent of the nucleated cells. In a properly-drawn and well-stained smear, there are very few 'disintegrating cells' and practically all the nucleated cells seen can be identified; in a highly active marrow, however, there are some cells which show mitotic division and are difficult to identify properly.

Table XV

Sternal puncture differential nucleated cell count

		Series I		Series II		
		Range	Mean	Range	Mean	Standard deviation
Nucleated red cells	Megaloblasts	0·0 to 1·5	0·7	0·0 to 3·25	0·92	± 0·7366
	Erythroblasts	0·4 to 10·0	3·4	0·0 to 3·3	0·49	± 0·6619
	Macroblasts			0·0 to 8·75	2·075	± 1·7675
	Normoblasts	16·0 to 25·5	21·6	7·5 to 63·2	24·17	±10·4505
White cell series	Myeloblasts	0·4 to 1·5	1·2	0·0 to 0·75	0·15	± 0·2670
A. Granular series	Pre-myelocytes	0·0 to 1·5	0·7	0·0 to 2·0	0·44	± 0·4409
	Myelocytes—					
	Neutrophil	1·0 to 9·5	4·4	1·0 to 18·75	8·30	± 3·2061
	Eosinophil	0·4 to 2·6	1·3	0·0 to 4·0	1·445	± 0·9498
	Basophil	None found.		None found.		
	Meta-myelocyte neutrophil	6·4 to 15·0	9·7	0·4 to 8·25	2·76	± 1·7257
	Staff or band neutrophil	14·6 to 38·5	25·3	12·4 to 60·0	33·91	± 9·1938
	Segmented neutrophil	6·5 to 25·6	16·8	0·0 to 23·6	5·35	± 5·0232
	Mature eosinophils	0·0 to 7·6	4·7	0·0 to 7·4	2·77	± 1·6539
	Basophils	0·0 to 1·0	0·2	0·0 to 1·2	0·08	± 0·2090
B. Non-granular series	Lymphocytes	3·0 to 12·0	6·25	2·9 to 34·5	15·68	± 7·2623
	Large mononuclears	1·2 to 7·0	3·0	0·0 to 3·5	1·585	± 0·9386
	Plasma cells	0·0 to 1·6	0·6	0·0 to 1·25	0·31	..

13

The Arneth and Schilling counts

Arneth (1904) divided the neutrophil polymorphonuclears into five main classes according to the number of segments in the nuclei which in their turn were classified into various groups according to the shape of the lobes. Under the original classification the count was very complicated and had only limited application in general practice. Cooke and Ponder (1927) modified and simplified the Arneth count taking into account only the five original basic divisions of the Arneth count without going into the intricate subdivisions of Arneth.

Schilling devised a method in which the differential leucocyte count and a simple nuclear lobe count of the neutrophils were incorporated and considered in the form of a 'hæmogram'.

Apparatus required (*vide* p. 76 *et seq.*).

Smears.—Smears for the Arneth and Schilling counts should be very thin and uniformly spread; they should be dried quickly by being waved to and fro or held under a fan (*vide* p. 77).

Staining.—Combined staining with May-Grünwald and Giemsa stains gives the most satisfactory result—the nuclei stain deeply and the connecting chromatin filaments are well shown, while the granules also show characteristic colour and size. If May-Grünwald and Giemsa stains are not available proper staining with Wright's or Leishman's stains, though not as satisfactory, gives quite good results. Iron-hæmatoxylin or hæmatoxylin staining for these counts in preference to any of the Romanowsky stains is sometimes advocated; while these stains bring out the nuclei and the chromatin filaments well, the colour of the granules is not distinctive so that differentiation between neutrophils and eosinophils is very difficult.

(A) Arneth count

The nucleus of the neutrophil polymorphonuclear cells is divided into lobes which may be separate or joined together by fine filaments of chromatin. Cooke's criterion of separate lobulation is that either the lobes should be distinctly separate, or should be joined together by a fine chromatin filament, but not by broad bands of nuclear material. Lobulation of the nucleus, it is assumed, gives an indication of the age of the cell—the fewer the lobes the younger is the cell. The youngest cell in the normal peripheral circulation is one which has a nucleus shaped like the letter C, while the cells with five or more distinct nuclei are the oldest. The principle of the method is to make a differential count of the neutrophil cells, to group the cells according to the lobulation of the nuclei, and to calculate the percentage of cells in each group.

The cells of the various types can be described as follows :—

Class I. Neutrophil granulocytes in which the nucleus has only one definite lobe, or two or more lobes joined by a definite band of chromatin and not by thin filaments.

Class II. When there are two separate lobes joined by a thin filament, or when there are two completely separate lobes.

Class III. When there are three separate lobes connected by thin filaments, or there are three completely separate lobes.

Class IV. When there are four separate lobes connected by thin filaments, or there are four completely separate lobes.

Class V. When there are five or more separate lobes connected by thin filaments, or there are five or more completely separate lobes.

When in doubt about the number of lobes in cells with three or more lobes, they must always be placed into the next lower class; for example, if there is any doubt whether a cell has three or four lobes, it should always be placed in class III.

The count is made by identifying and classifying one hundred consecutive neutrophil granulocytes from different parts of the slide, and the result is usually reported in the following form :—

Example 1, *a normal count* (*European standard*).

Class	..	I	II	III	IV	V	Total.
Number	..	10	25	47	16	2	100

When there is an increase in the number of cells with one and two separate nuclear lobes, i.e. of class I or class II, at the expense of those with three or more lobes, there is said to be a 'left shift' in the Arneth count, as in the example given below :—

Example 2, *a* '*left shift*' *count*.

Class	..	I	II	III	IV	V	Total.
Number	..	41	44	14	1	0	100

Conversely, when there is an increase in the 4- and multi-lobed nuclei, it is known as a 'right shift'.

Example 3, *a* '*right shift*' *count*.

Class	..	I	II	III	IV	V	Total.
Number	..	9	22	36	23	10	100

Various indices and methods of expressing more concisely the results of the Arneth count (Cooke's modification) have been proposed. Probably the most suitable of these methods is the calculation of the weighted mean of the nuclear lobes; this is obtained by multiplying the number of cells in class I by 1, the number in class II by 2, the number in class III by 3, the number in class IV by 4, the number in class V by 5, and then adding together and dividing the sum total by the number of cells counted.

The weighted means of the nuclear lobes in these three examples are calculated in the following way :—

Example 1	Example 2	Example 3
1 × 10 = 10	1 × 41 = 41	1 × 9 = 9
2 × 25 = 50	2 × 44 = 88	2 × 22 = 44
3 × 47 = 141	3 × 14 = 42	3 × 36 = 108
4 × 16 = 64	4 × 1 = 4	4 × 23 = 92
5 × 2 = 10	5 × 0 = 0	5 × 10 = 50
100 275	100 175	100 303
Weighted mean = 2·75	1·75	3·03

Interpretation.—Immature granulocytes and cells of Arneth class I and some of class II, but none of class III, IV or V, are found in the bone marrow. If there be a sudden stimulus to the bone marrow to produce granulocytes, as in an infection, the leucocytosis is brought about by young cells appearing in the blood, the number and the proportion of these depending upon the nature of the stimulus and the reacting power of the marrow of the individual and there is a shift to the left in the Arneth count. But leucocytosis as a result of muscular exercise is only due to redistribution of cells, is not accompanied by any increase of young leucocytes in the peripheral circulation, and is therefore not associated with any 'left shift'.

Again, in chronic infection or toxæmia, there is an increased leucocyte destruction, which may or may not be balanced by new leucocyte formation ; in the former case there will be leucocytosis or a normal count and in the latter leucopenia. In either case there is an increase in young forms, actual or relative, which results in a shift to the left and a decrease in the weighted mean. It will be apparent that this shift is quite independent of the total leucocyte or total granulocyte count, and provides another indication of the degree of the toxæmia.

In pernicious anæmia and certain other conditions of bone marrow dysfunction, there is a shift to the right in the Arneth count, and therefore an increase in the weighted mean ; in such conditions there are many multi-lobulated cells and only very few young cells coming into the circulation.

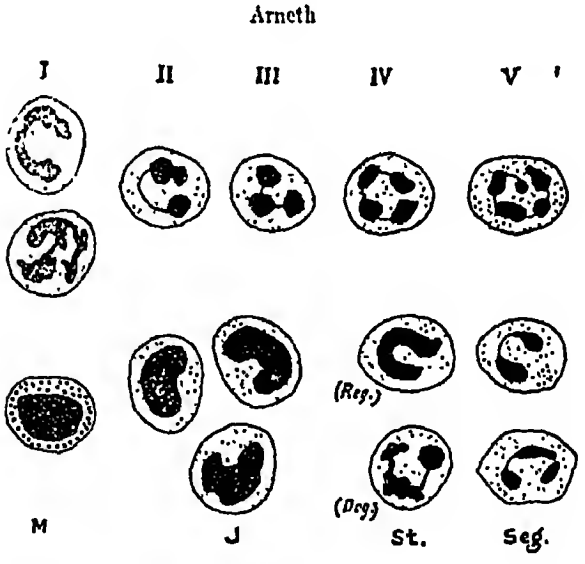
Normals

	I	II	III	IV	V	Weighted mean
Cooke and Ponder (1927) ..	10	25	47	16	2	2·75
Kennedy (1933) (Iraq) ..	13	30	43	10	4	2·62
Das Gupta (Calcutta) ..	37	44	16	3	0	1·85

(B) Schilling count

In this count the neutrophil granulocytes are divided into four groups and classified as follows:—

1. Myelocytes (M).
2. Juveniles (J); these have a distinct indentation in the nucleus; they correspond to our (*vide* p. 91, table XI) meta-myelocytes.
3. Stab cells (St); these have a C- or horse-shoe-shaped nucleus but no true lobulation; they correspond to class I of the Arneth count, and to our staff cells.



Schilling

Stab cells may be of the regenerative or degenerative type, and are differentiated by the shape and staining reaction of the nucleus; in the latter the nucleus is ribbon-like and pycnotic with clumps of chromatin, and usually irregular shaped, and the cytoplasm may be vacuolated.

Segmented (Seg.) polymorphonuclears, showing two or more distinct lobes.

According to Schilling the following are the constituents of a normal hæmogram:—

Total W. B. C.	Percentages							
	B.	E.	M.	J.	St.	Seg.	L.	Mon.
5,000 to 8,000	0–1	1–2	0	0–1	3–5	57–67	21–35	4–8
			60–70					

B. = basophil. E. = eosinophil.
L. = lymphocyte. Mon. = large mononuclears.

Discussion.—The Schilling hæmogram is much more than a differential granulocyte count, as is the Arneth count, and Schilling claims that it gives far more information. The interpretation depends on a highly imaginative series of responses to infection and toxæmia that are supposed to, and possibly do, occur in the hæmopoietic system, in which there are three phases, neutrophilic struggle, monocytic defence, and lymphocytic cure, but it does not seem to take into account the nature of the infection and the consequent variations in the response. Further, there are so many directions in which changes may take place that the hæmogram seems peculiarly susceptible to facile interpretation to suit the conscious or unconscious wishes of the interpreter.

We do not propose to give a full discussion on the interpretation of the hæmogram, as it has only an indirect bearing on the subject of anæmia, but as far as the neutrophil granulocyte elements are concerned Schilling's contention is that infection may cause two types of reaction, the regenerative reaction and degenerative reaction; in the regenerative reaction, new leucocytes are formed in the bone marrow and this is reflected in the peripheral circulation by a rise in the total white cell count, an increase in the juveniles and regenerative forms of stab cells, while in the degenerative reaction, which is found in severe toxæmia, on account of the degenerative influence of the toxin on the marrow, the formation of new cells is retarded; this may be shown by a leucopenia. In the differential count of such a case, there are few juvenile cells and the stab cells that reach the circulation fail to segment at the usual rate, and may degenerate with the result that there is a preponderance of stab forms, including many degenerative forms, in the blood. The reaction, however, may not be purely regenerative or degenerative in character, but may be a mixture of the two, and the hæmogram may be intermediate, with slight leucopenia and a greater preponderance of the younger cells than in a purely degenerative process.

(It is surprising to the writers that no provision is made in the classical Schilling hæmogram for the separate counting of the regenerative and degenerative forms of stab cell, when obviously the presence or absence of the latter is an important factor in the interpretation of the hæmogram.)

Conclusion.—The interpretation of the Arneth count is simpler, though the implications are more limited, but it presents a minor practical objection in that more skilful staining is required to ensure accurate counting of the separate lobes.

The value of these counts is mainly in establishing the presence of infective or toxæmic factors, which may be important contributory causes of the anæmia, and/or may prevent response to appropriate hæmatinics. The presence of multilobular cells, shown only in the Arneth count, is important in the diagnosis of pernicious anæmia.

14

The erythrocyte sedimentation rate

The determination of the erythrocyte sedimentation rate (ESR) is not a test that gives much direct information regarding the nature of the anæmia, but it is of value in differentiating 'functional' disorders, such as pernicious anæmia in which the true ESR is within normal limits, from anæmia due to such causes as sepsis and malignant disease in which the ESR is markedly increased.

Further, the ESR is affected by changes in the shape and size of the red cells, in so far as these changes affect rouleaux formation, and by changes in the concentration of the red cells in the plasma; this last may overshadow the other changes and, being a measurable factor, must be allowed for in estimating the true sedimentation rate.

Finally, as from the single specimen taken in the normal course of a routine blood examination the ESR can be estimated without additional labour, it should always be included.

The test suffers from the multiplicity of the methods by which it is performed and by the variety of the forms in which the results are expressed. The most commonly used technique is that of Westergren (1926), but, as it is important that the correction for anæmia should be made and as no correction chart has been worked out for this technique, this method has its limitation.

Wintrobe's method (1933) is really a modification of this method; it has the advantage that the cell volume can be estimated on the same sample of blood and also that a correction for anæmia can be made.

Principle of the test.—A known volume of blood is intimately mixed with a definite amount of anti-coagulant, and the mixture is drawn into a tube of known calibre up to a certain point; the tube is then allowed to stand in an upright position at room temperature and the level of the red blood cells in the tube is noted at the end of one hour and again after two hours. The fall in the level of the red cells is expressed in millimetres.

Westergren's method

Apparatus required

(*i*) Sedimentation tubes.

Westergren tube. This is about 300 mm. long and is graduated from 0 to 200 mm. in 1 mm. intervals; it has a uniform bore of about 3 mm. and is open at both ends.

(*ii*) Special Westergren rack, or an improvised rack, to keep the tubes in a vertical position, and to prevent the blood escaping.

(*iii*) Anti-coagulant—3·8 per cent sodium citrate solution.

(*iv*) Syringe, etc., for collection of blood.

(*v*) Stop watch.

Technique.—In this method 3·8 per cent solution of sodium citrate is used as anti-coagulant in the proportion of one part of citrate solution to four parts of the blood.

Put exactly 2 c.cm. of blood into a flask to which 0·5 c.cm. of 3·8 per cent solution of sodium citrate has already been placed. Mix immediately by rotating the flask and subsequently if possible in a shaker; then draw up the mixture into a Westergren's standard tube to the zero mark which is exactly 200 mm. from the tip. The tube is now set upright in a stand in which a spring clip holds the points of the tube firmly against a rubber cork at the bottom end. The tube is now left to stand and the upper level of the red cell column, which is generally sharp, is read at the end of one and again after two hours*. The length of the column of plasma from the zero mark to the top of the red cell column is the amount of sedimentation and this is read off in millimetres; this is reported as the sedimentation rate in millimetres for one hour and for two hours.

Normal limits (after one hour)—

	Men	Women
Textbooks	3 to 5 mm.	4 to 7 mm.
Our own experience in Indians in Calcutta.	3 to 15 mm.	5 to 40 mm.

The high figures that we obtained in apparently normal subjects are partly explained by the higher temperatures that prevailed, compared with those of the places where the tests on which other normal figures are based were carried out.

Wintrobe's method

Apparatus required

(*i*) Wintrobe's tubes—a flat-bottomed glass tube 115 mm. long graduated from 0 to 100 in 1 mm. intervals; it has a uniform bore.

(*ii*) Anti-coagulant—sodium and potassium oxalate mixture (*vide* p. 36).

(*iii*) Electric centrifuge.

Together with a syringe, etc., and stop watch as in Westergren's method.

Technique.—Wintrobe used his cell-volume tube for the determination of the sedimentation rate.

* Only one-hour readings are usually recorded.

The blood is collected in an 'oxalated' flask and is thoroughly mixed; the cell-volume tube is filled with the oxalated blood by means of a capillary pipette up to the zero mark, which is exactly 100 mm. from the bottom. The tube is now allowed to stand upright in a vertical position on a special stand or on a tray of plasticine, and the reading is taken at the end of one and again after two hours, in the same way as in Westergren's method; the cell-volume tube is then put into a centrifuge and centrifuged at 3,000 revolutions per minute for half an hour to obtain the packed-cell volume percentage; thus both procedures are done in the same tube with the same blood sample.

Normal limits (after one hour)—

	Men	Women
Textbooks	0 to 9 mm.	0 to 20 mm.
Our own experience in Indians in Calcutta.	2 to 20 mm.	2 to 30 mm.

Correction for anæmia.—One important factor in the rate of sedimentation of bodies in fluid is the concentration of these bodies. The sedimentation standards for red cells in plasma are based on there being a normal number of red cells in a given volume of blood, and in anæmia when these are reduced the sedimentation

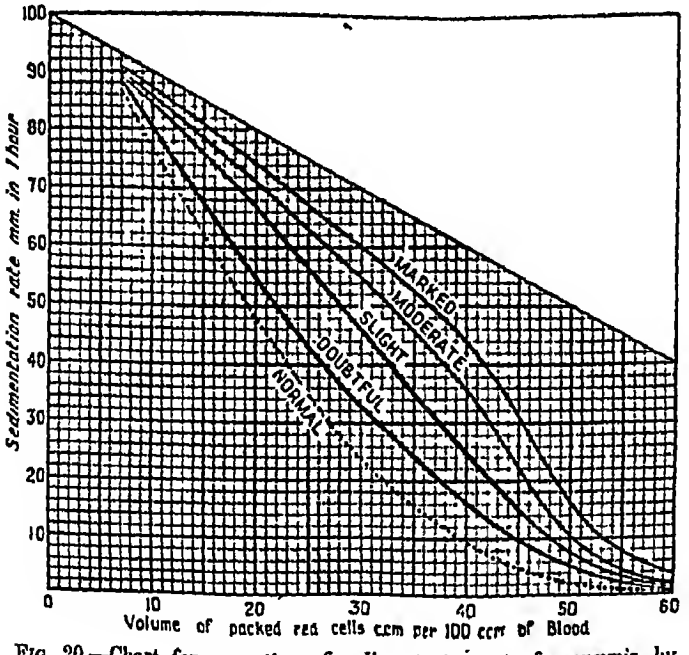


FIG. 20.—Chart for correction of sedimentation rate for anæmia by means of the corpuscular volume.

rate is increased above the normal, irrespective of the other changes in the blood. To make the correct allowance for this fact Hynes and Whitby (1938) worked out a chart showing the relationship of cell volume and sedimentation rate in normal blood and in blood showing various degrees of anæmia; this chart is reproduced above.

To correct for anæmia, find the junction of the lines of the observed sedimentation rate and the observed corpuscular volume in the chart; this point will fall in one of the five zones (normal, doubtful, etc.) which indicate the approximate degree of increase in the rate. If a 'compensated' figure is required, follow the appropriate curve down to the point where it cuts the 45 c.cm. vertical lines, for men, and the 40 c.cm. line for women.

Example.—Observed rate = 50 mm.; observed corpuscular volume = 30 per cent; point of junction lies in area of 'slight' increase; compensated rate for men = 18 and for women = 28.

Precautions

(*i*) All apparatus must be scrupulously clean and dry.

(*ii*) The dilution with sodium citrate solution in Westergren's method should be absolutely correct.

(*iii*) The specimen of blood should be collected during the fasting state and examined within one hour of collection.

(*iv*) In women it is advisable to avoid the menstrual period and the few days before and after it.

(*v*) The specimen should be rejected if there is a slight trace of coagulation or any marked hæmolysis.

(*vi*) The tubes should be set up in an absolutely vertical position in a quiet place, well away from any apparatus that vibrates. The temperature of the room should be between 72° and 80°F. whenever possible.

(*vii*) In recording the results, correction for anæmia must always be applied.

Discussion.—There are a number of factors involved in the phenomenon of erythrocyte sedimentation, but the most important is the size of the rouleaux formation and this is mainly determined by the composition of the plasma. Increase in plasma fibrinogen will cause an increase in the size of the rouleaux formation and therefore an increase in the sedimentation rate. It is probably on this fact that the value of this test depends, as, with the exception of the concentration of the red cells for which allowance can be made, this effect overshadows all others.

The sedimentation rate is increased in so many conditions that it is very dangerous to attach any specific diagnostic significance to it. This must be quite obvious from the wide range of the ESR rates that has been recorded in apparently healthy individuals. However, the ESR is a test of great value in estimating the progress in chronic infections, such as tuberculosis and leprosy. A standard technique should be adopted, and in reporting the results the method employed must be stated or the information loses much of its value.

15

Gastric analysis

Introduction.—The gastric juice in the normal individual contains hydrochloric acid, free and in the combined state, the enzymes pepsin and rennin, and the 'intrinsic factor of Castle'. Examination of the aspirated gastric juice, primarily for acidity, but also for the presence of the enzymes, and for other normal and abnormal characters, is known as gastric analysis.

We have included gastric analysis in this series on hæmatological technique, because normal hæmopoiesis is to a large extent dependent on normal gastric function, and consequently the knowledge that we obtain from this test is important in both the diagnosis and the treatment of the anæmias.

Achlorhydria or hypochlorhydria are associated with deficient digestion and absorption of a number of food substances. In some cases of microcytic anæmia, achlorhydria is considered to be the main ætiological factor; iron is more easily absorbed from an acid than from an alkaline medium. Free acid is necessary also for ensuring proper peptic digestion (*v.i.*). Further, in the absence of hydrochloric acid, fermenting organisms flourish, causing flatulence, meteorism, and diarrhœa. This mucosal dysfunction leads to a macrocytic anæmia; the anæmia of sprue is often of this type and due to this cause; but neither in sprue nor in nutritional macrocytic anæmia is achlorhydria constant, nor is there any evidence that the 'intrinsic factor' is also absent. Finally, in pernicious anæmia there is complete and constant achylia*, which is associated with the absence of the 'intrinsic factor' though this latter deficiency cannot be demonstrated directly in the laboratory.

In practically all cases of anæmia associated with achlorhydria or hypochlorhydria, the giving of dilute hydrochloric acid, alone or with pepsin, will be beneficial, and may in fact constitute an important part of the treatment.

Methods: Gastric analysis can be done by either of the following methods:—

(*i*) Single examination: This is an old method and is almost obsolete now. In this method, after complete evacuation of the fasting juice, an Ewald meal, consisting of bread and water, is given to the patient and the stomach contents withdrawn again after one hour.

(*ii*) Fractional analysis of Rehfuss: In this method, after complete evacuation of the fasting juice, a suitable test meal is given to the patient; small samples, about 10 c.cm., of the gastric fluid are drawn off every 15 minutes up to 2½ to 3 hours.

---

* The following terms are in common use for the different stages of gastric deficiency:—

Achlorhydria (or false achlorhydria).	= absence of free hydrochloric acid during an ordinary test meal, in which histamine was not given.
Complete achlorhydria	= absence of free acid even after histamine injection.
Achylia	= absence of free acid and the gastric enzymes, even after histamine.

Fractional gastric analysis gives valuable information as to the motility and secretory functions of the stomach. This method is followed by us in the investigation of all cases of anæmia in the hospital and in many of the cases attending the anæmia outdoor clinic.

In the course of the fractional gastric analysis, an injection of 0·5 mg. of histamine is given one hour after the test meal to the patients who fail to show any free acid in any of the previous specimens.

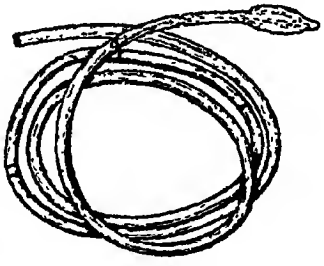


FIG. 21.

Apparatus required

(*i*) Rehfuss' or Ryle's tube, or any suitable modification (*vide* figure 21). These are tubes made of good rubber. They are about 32 inches long with an internal diameter of about 1/8th inch. One end of the tube is open, while the other, the tip, ends in an olive-shaped bulbous protuberance filled with metal to weight it and keep it rigid. There are holes in the rubber at or near the tip for the passage of the gastric juice through the tube. The distance up to which the tube has to be passed is shown by a mark near the open end, about 20 to 22 inches from the tip.

Before use the tube is sterilized in boiling water for a minute or two, and is kept coiled up in a sterilized petri dish until required.

(*ii*) Record syringes 10 c.cm. and 1 c.cm.

(*iii*) Twenty-five c.cm. burette graduated in 1/10th of a c.cm. with a stand.

(*iv*) Twelve labelled test-tubes in a rack, *plus* a few extra tubes.

(*v*) Capillary pipettes with teats.

(*vi*) Small glass funnel.

(*vii*) Porcelain dishes—3.

(*viii*) Glass rods—3.

(*ix*) Centrifuge machine.

(*x*) Slides.

(*xi*) Microscope.

Chemicals required

(*i*) One per cent of cocaine, or a suitable substitute.

(*ii*) Seven per cent alcohol 100 c.cm.

(*iii*) N/10 sodium hydroxide.

(*iv*) Dimethyl-amido-azobenzene 0·5 per cent in 95 per cent alcohol (Töpfer's reagent).

(*v*) Phenolphthalein 1 per cent in alcohol.

(*vi*) Histamine—0·5 mg. in 0·5 c.cm. sterilized distilled water.

(*vii*) Adrenaline chloride solution, 1 in 1,000 for injection.

(*viii*) Sulphur powder.

(*ix*) Benzidine.

(*x*) Glacial acetic acid.

(*xi*) Hydrogen peroxide.

(*xii*) Pepsin.

(*xiii*) Mett's albumin tubes (*v.i.*).

(*xiv*) Dilute hydrochloric acid.

(*xv*) Fresh milk.

(*xvi*) Lugol's iodine solution.

ROUTINE PROCEDURE

The following is our usual procedure in carrying out a fractional gastric analysis:—

A. Preparation of the patient

On the previous night, the patient has his usual meal at 8 p.m., and at 10 p.m. he is given four charcoal tablets with a glass of milk. The next morning he is not

allowed any food or drink before the test is finished. Indian patients must be warned not to chew any *pan*, as it may impart a red colour, which might be mistaken for blood, to the gastric juice.

Before introducing the tube, in very sensitive patients, the nasal mucous membrane and the posterior part of the pharynx may be sprayed or swabbed with 1 per cent solution of cocaine, or with a suitable substitute.

B. Introducing the tube

(*i*) Through the mouth: With the patient in a comfortable position, sitting if possible, but the test can be done with the patient lying down; the tip of the tube is placed on the posterior portion of the dorsum of the tongue and allowed to drop slowly backwards and downwards. When the tip strikes the posterior pharynx, the patient is instructed to make swallowing movements and the tip will then pass into the œsophagus. The patient should be told to continue to swallow slowly, when the tube will gradually descend until the end has reached the fundus of the stomach; the mark on the tube gives an approximate indication of when this point is reached.

(*ii*) Introducing through the nose: With a little practice, the introduction of the tube through the nose is much easier than through the mouth and can be carried out even in the most sensitive patient. The tip of the tube is introduced into one of the nasal orifices and gently pushed through the nose until it reaches the posterior wall of the pharynx. The patient is now told to make swallowing movements while the tube is pushed gently down until it reaches the fundus of the stomach.

Some obstruction may be felt in passing the tube through the nose; this is easily overcome by a little manipulation, but if the resistance is great the tube must be taken out and introduced through the other nasal orifice, as not infrequently the septum is deviated to one side.

C. Drawing out the contents of the fasting stomach

When the tube has been introduced up to the required distance, introduce the nozzle of a 10 c.cm. syringe into the tube, with the piston drawn out; push down the piston so that the air in the syringe is forced through the tube, to dislodge any mucus or food debris that may be blocking it at its distal end; then aspirate the contents of the fasting stomach. If there is any difficulty in getting the juice, vary the position of the tube in the stomach by drawing it out or pushing it in, and/or by forcing more air through the tube.

Rarely, difficulty in obtaining juice may be due to contraction of the gastric muscles, which may be difficult to overcome. An attempt should be made to draw out all the fluid of the fasting stomach—by altering the position of the tube in the stomach, by putting the patient in different postures, and by applying a little pressure to the stomach from outside. The contents of the stomach are placed in the test-tubes previously labelled.

After complete evacuation of the stomach contents the test meal is given to the patients with the tube *in situ*.

D. Test meals

An ideal test meal is obviously one that bears a close similarity to the ordinary diet of the patient, but, for many reasons, it is not possible to give such a test meal. Various test meals have been advocated, but here we shall describe only two with which we have had personal experience.

(*i*) Gruel test meal: In making this we have always used Quaker oats, but any form of prepared oats can be used, and in this country some workers prefer to use a rice gruel. Take a tablespoonful of Quaker oats in two pints of water, add a pinch of salt, boil down to a pint, and strain through fine muslin. This meal does not contain any lactic acid and is thus almost an ideal test meal, but, with the tube *in situ*, it becomes very difficult and sometimes impossible to swallow such a large quantity of thick gruel.

(*ii*) Alcohol test meal: One hundred c.cm. of 7 per cent alcohol is used for this meal. The measured quantity of alcohol is placed in a beaker from which it is drawn up into a syringe and introduced into the stomach through the tube; the process is repeated until the whole amount has been introduced.

This meal is very easy to administer, while the fluid that is subsequently withdrawn is almost clear; this allows of easy titration for acidimetry.

E. Withdrawal of post-prandial specimens

Note the time when the test meal is given, aspirate with a syringe about 10 c.cm. of gastric contents every 15 minutes up to 2½ or 3 hours. The specimens are kept in labelled test-tubes until the time of examination.

Histamine.—As the fasting and post-prandial juices are withdrawn, they are examined for the presence of free hydrochloric acid by the bedside.

Take one c.cm. of the gastric juice in a small test-tube, add a small drop of Töpfer's reagent. Note the colour—red or orange colour indicates the presence of free hydrochloric acid and further bedside examination of subsequent specimens is not necessary.

If free hydrochloric acid is not present in the fasting juice and in the first four post-prandial specimens, an injection of 0·5 mg. of histamine is given and the procedure of withdrawing samples is continued as before. A little flushing of the face is seen after injection of histamine, and occasionally the patient may complain of palpitations which generally pass off quickly. In the event of the patient becoming distressed by these symptoms, an injection of 0·5 c.cm. adrenalin chloride should be given; this will give instantaneous relief.

Examination of Gastric Contents

A. Macroscopic examinations

(*a*) *In the fasting juice.*

(*i*) Amount: measure and note the amount. Normally 20 to 25 c.cm. are found. Marked increase over 50 c.cm. suggests hypo-motility, obstruction, or hypersecretion.

(*ii*) Odour: normally it has no striking odour. An offensive odour suggests cancer, and a sour odour fermentation.

(*iii*) Remnants of food or charcoal: normally no food remnants or charcoal are found after 10 hours' interval. The presence of food remnants or charcoal particles suggests hypo-motility, pyloric obstruction, or ptosis.

(*b*) *In the fasting juice and in the post-prandial specimens.*

(*iv*) Mucus in large quantity in the fasting juice, and in many of the later post-prandial specimens, indicates catarrhal gastritis.

(*v*) Bile: traces of recently regurgitated lemon-yellow bile may be seen in a few specimens and are usually due to retching caused by the introduction of the tube. A large quantity of turbid green bile in the fasting and in any of the early post-prandial samples is almost always pathological.

(*vi*) Blood: macroscopic examination for blood gives more valuable information than the chemical examination. Flecks of fresh blood are usually the result of trauma in passing the tube, while large quantities of fresh blood in any specimen would indicate varices, erosions, or even malignant ulceration of the œsophagus. Blood from a gastric ulcer or carcinoma of the stomach is changed to brown acid hæmatin by the acid in the stomach juices; it is found most frequently in the fasting juice, but it may be found also in any post-prandial specimen.

B. Chemical examinations

(*i*) *Acidimetry.*

Place 5 or 10 c.cm. of clear gastric contents in a shallow porcelain dish. If there is an excess of mucus in any specimen, filter the gastric juice through a plug of cotton-wool to remove the mucus; this will then allow of easy titration.

Add a drop of Töpfer's reagent to the gastric juice in the porcelain dish, the presence of free hydrochloric acid will be indicated by the red or orange colour of the juice.

(*a*) *Estimation of free hydrochloric acid*

Titrate with N/10 sodium hydroxide until the red or orange colour is discharged: this is done as follows:—

Fill a graduated 25 c.cm. burette with N/10 NaOH up to the zero mark. With one hand regulate the flow of NaOH and allow it to fall drop by drop from the burette into the porcelain dish; stir all the time with a clean glass rod with the other hand. The end point will be shown by a brownish and not a yellow colour. Take the reading of the burette, the difference between the two readings (the first should be zero) gives the amount of NaOH that was required to neutralize 5 or 10 c.cm. of gastric juice.

In clinical work the result is always expressed as the number of cubic centimetres of N/10 NaOH which would be required to neutralize 100 c.cm. of the gastric juice, each cubic centimetre representing *one degree* of acidity. So, the number of degrees of acidity is calculated by multiplying the number of cubic centimetres of N/10 NaOH by 10, if 10 cubic centimetres of gastric juice was used, or by 20, if 5 c.cm. of gastric juice was used.

The result may be expressed in grammes of hydrochloric acid by multiplying the number of degrees of acidity by 0·00365 (an easy way to remember this is that 365 is the number of days in the year, the last figure—5—indicating the number of decimal places).

Example.—If 10 c.cm. of gastric juice was taken and if the end point was reached when 2·3 c.cm. of N/10 NaOH had been added, the degree of acidity is 2·3 × 10 = 23.
And in terms of hydrochloric acid 23 × 0·00365 = 0·08395 g. of hydrochloric acid.

(*b*) *Estimation of total acid*

After the estimation of free hydrochloric acid, or if there is no free HCl at all, 1 or 2 drops of phenolphthalein (1 per cent solution in alcohol) is added as an indicator and the titration with N/10 NaOH is continued until a permanent faint red colour develops.

The third reading *minus* the first reading (zero in this case) multiplied by 10, if 10 c.cm. was taken, or 20, if 5 c.cm. was taken, gives the degree of total acid.

The total acidity is made up of free HCl together with the HCl which is combined with protein and mucus, *plus* the organic acids, such as lactic and butyric, which result from fermentation.

The estimation of total acidity is of little practical importance. It is usually about 10 degrees higher than the free HCl, except in cases of marked lactic or butyric acid fermentation, when it may be very much higher than the free HCl. In these cases the typical 'rancid butter' odour enables one to detect fermentation.

(c) *Lactic acid*

Test for lactic acid must be applied in all cases where there is no free HCl in the fasting juice.

Add 2 drops of 5 per cent aqueous solution of ferric chloride to half an inch of clear gastric fluid in one tube and to half an inch of water in another tube of the same size which will serve as a control. To each add 6 drops of saturated solution of mercuric chloride. If lactic acid is present in the gastric contents a deep yellow colour will be produced in that tube, but not in the other.

(*d*) *Bile*

The presence of bile will be denoted by the colour in the gastric fluid. If in doubt. Hay's sulphur test can be done, by sprinkling sulphur dust on the surface of a specimen of clear juice. If bile salts are present, the sulphur will fall to the bottom.

(*e*) *Blood*

Dissolve a little benzidine in 2 c.cm. of glacial acetic acid, warm if necessary; add 2 c.cm. of 3 per cent hydrogen peroxide. Finally, add 1 c.cm. of the gastric juice, and mix well by shaking. A blue or bluish-green colour indicates the presence of blood.

The test is very sensitive and will detect blood in dilution of 1 : 3,000,000.

C. Examination for enzymes

(*i*) *Detection of pepsin.*—Normally pepsinogen is secreted by the stomach, and is transformed into pepsin by the free HCl in the stomach. Its presence is detected by the digestion of egg-albumin.

Method.—Take three small test-tubes.

In tube 1, put 1 g. of pepsin, 2 c.cm. of the HCl solution and a one-inch length of capillary tube of albumin*.

In tube 2, put 2 c.cm. of clear gastric fluid and a capillary tube of albumin.

In tube 3, put 2 c.cm. of clear gastric fluid, 2 c.cm. of the HCl solution and a capillary tube of albumin.

Place all the three test-tubes in a warm incubator overnight, examine them for digestion of the albumin next morning.

Tube 1 in which digestion should be complete serves as a control.

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* Mott's capillary tubes of albumin are prepared in the following way:—

Mix the white of 3 or 4 eggs, beating them gently.

Make some capillary tubes of uniform bore. Fill the tubes with the egg-albumin and cut them into pieces of about 10 to 12 inches long. Seal the ends with sealing wax. Boil a large quantity of water in a big vessel. Just as the water begins to boil, put the capillary tubes containing albumin into the boiling water. Remove the vessel from the source of the heat. After 5 minutes take out the capillary tubes—the egg-albumin will be lightly coagulated. Store the tubes in a refrigerator.

Before use, cut the egg-albumin tubes into lengths of one inch, taking care that the ends are clean cut and circular.

Interpretation.—Digestion in tube 2 indicates the presence of both pepsin and free hydrochloric acid in the gastric juice.

If digestion fails in tube 2 but occurs in tube 3, pepsinogen is present, but it required the free HCl to be added to the tube to convert it into pepsin.

If there is no digestion even in tube 3, then both pepsin and pepsinogen are absent.

Partial digestion either in tube 2 or 3 denotes the presence of pepsin or pepsinogen in small amount.

(*ii*) *Detection of rennin.*—The enzyme rennin coagulates the protein of milk. Fresh milk is used as the reagent.

Neutralize 5 c.cm. of clear gastric fluid with very dilute sodium hydroxide. Add an equal amount of fresh milk and place in a water-bath at 40°C. for 15 minutes.

Interpretation.—Coagulation of the milk in 10 to 15 minutes denotes the presence of a normal amount of rennin; delayed coagulation denotes a decreased amount.

D. Microscopic examination of the gastric residue

This examination has very limited application and need only be done if carcinoma is suspected. The fasting contents when no free HCl is present are most suitable for the test.

Centrifugalize the fasting contents, take a small loopful from the bottom and smear it on a glass slide. Fix by heat and stain by Lugol's iodine and examine with the oil-immersion lens for 'Boas-Oppler' bacilli.

'Boas-Oppler bacilli are large (5 to 10μ long), non-motile and usually arranged in clumps, or end to end in zig-zag chains. They stain yellow to brown with iodine solution, which distinguishes them from *Leptotrichia buccalis*, which is not infrequently swallowed, and hence found in stomach fluid' (Todd and Sanford, 1939).

Method of recording results

The results are best recorded on a special chart which may be printed or cyclostyled.

Figure 22 shows the form of chart that we use, except that in this figure a shaded area has been added to show the range within which 80 per cent of gastric analyses in 'normal' individuals fall.

Both free acid and total acid are recorded in the chart, preferably in different colours. The presence of mucus, bile, and blood is indicated by a *plus* or a double *plus* sign in the appropriate place.

Normal standards

Acid curves will vary not only in height but in shape, and one cannot judge them solely on one feature, e.g. on the highest point reached during the test. Nevertheless, if a curve is to be classified on a single feature this is probably the

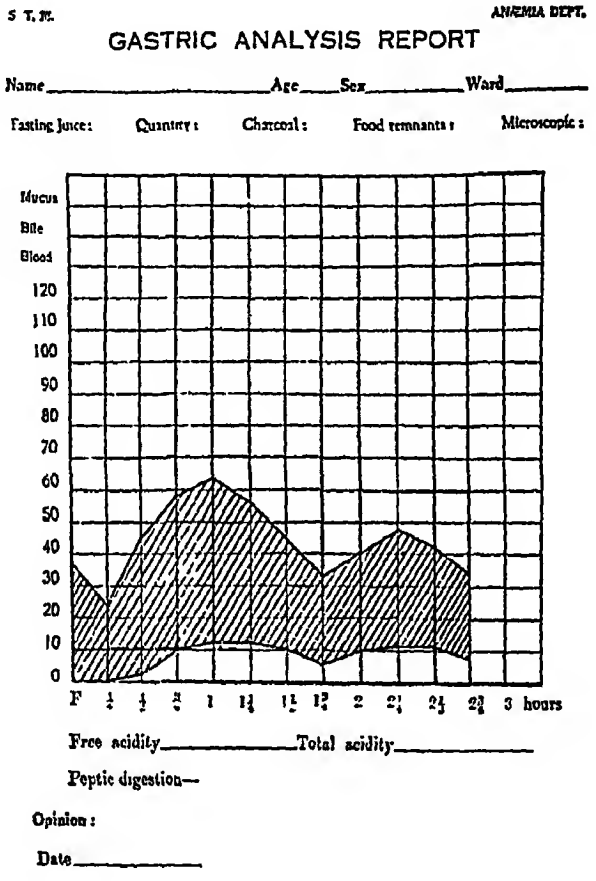

FIG. 22.

best one, as it usually reflects fairly accurately the rest of the curve. We have adopted the following criteria for classifying acid curves, according to the highest free-acid reading :—

	c.cm. of N/10 NaOH required to neutralize 100 c.cm. gastric juice
Achlorhydria	0
Hypochlorhydria	<10
Isochlorhydria, low	10 to <25
,, medium	25 to 45
,, high	> 45 to 65
Hyperchlorhydria	>65

These criteria are based on our personal experience in India (Napier and Das Gupta, 1935; Napier, Chaudhuri and Rai Chaudhuri, 1938) but they do not differ materially from those adopted by workers in other countries. About 80 per cent of 'normal' individuals fall within the isochlorhydria range; within this range women will usually be lower than men (figure 23). There is no evidence that the gastric acidity of Indians in India is lower than that of Europeans in their own countries. The data available from our own experience suggest that in South Indians and Bengalis (figure 24) the acid range is a little higher than the textbook figures which are based on Europeans and North American subjects.

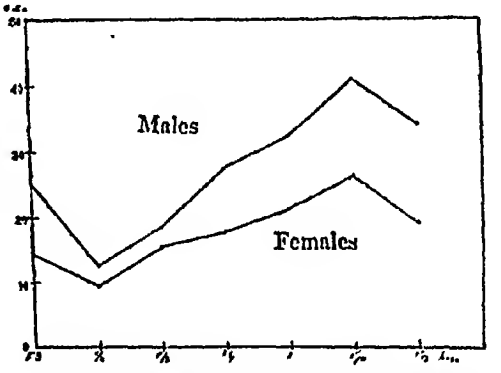

FIG. 23.—Assam coolies: males and females (gruel test meal).

From 2 to 4 per cent of apparently normal Indians are completely achlorhydric even with histamine; this is in keeping with experience in other countries.

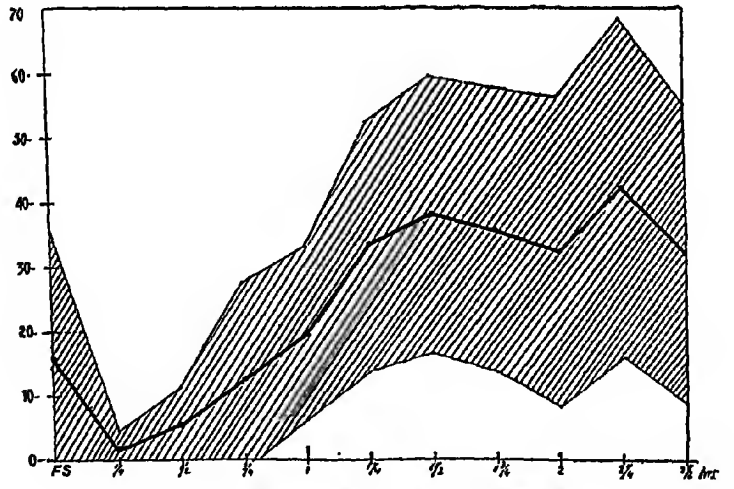

FIG. 24.—Normal Indians in Calcutta (gruel test meal).

With the alcohol test meal the acid curve on the whole tends to be higher; this is particularly noticeable in the first post-prandial sample in which the initial drop in acidity, due mainly to the neutralization of the acid by the meal, may be absent. Another difference is that the maximum acidity is usually reached earlier when alcohol is given as a test meal.

Other indications given by gastric analysis

In chronic gastritis, in the early stages there is hyperacidity and hypersecretion; this latter is shown by the large quantity of fasting juice, but in the later stages there is gastric atrophy with accompanying hypoacidity or achlorhydria.

In gastric ulcer there is usually increased acidity and blood may be present.

In gastric carcinoma, the findings are sometimes very characteristic, but in the early stages there may be no indications, so that it is dangerous to exclude carcinoma on the strength of a normal gastric analysis. There is achlorhydria, excess of lactic acid, and usually blood, and the Boas-Oppler bacillus is often present. If the cancer is at the pylorus there may be delayed emptying and food or charcoal will be found in the fasting juice which will usually be very sour and offensive.

In gastric neuroses there is usually hypersecretion, and in dilatation of the stomach food retention and fermentation.

16

The red cell fragility test

Introduction.—When normal red cells are suspended in 0·85 per cent saline they remain unaltered, but they become crenated when the concentration of the saline is raised above 0·9 per cent, whereas they are hæmolysed, or 'laked', when the concentration is lowered below 0·45 per cent.

The study of the reaction of the red cells in hypotonic saline solution is known as the fragility test of the red cells. The test can be done by either a qualitative or a quantitative method; it is conveniently done with fresh oxalated venous blood collected for routine examination of the blood (p. 35), but it can also be done with ordinary (non-oxalated) venous blood, or with the capillary blood from the finger.

The fragility of the red cells is expressed in terms of percentages of sodium chloride solution, namely, the points at which hæmolysis commences and at which it is complete.

Qualitative method

To each of a series of numbered test-tubes containing saline solution of different graded concentrations, one drop, or two drops of blood are added; the contents of the tubes are mixed well and allowed to stand for about 2 hours, after which the numbers of the two tubes that show the beginning of hæmolysis and complete hæmolysis are noted, and from these the strength of the corresponding saline solutions are calculated.

Apparatus and reagents required

(*i*) Accurate solutions of sodium chloride, 0·5 per cent and 1·0 per cent, standardized against silver nitrate solution.
(*ii*) Small test-tubes.
(*iii*) Metal rack for holding the tubes.
(*iv*) 5 or 10 c.cm. syringe with needle of fairly wide bore.

A convenient form of rack is that used for the Wassermann reaction. The holes are in two rows, with single tubes at either end of the rack. The holes are numbered 12 to 24 and those at each end 'S' and 'W' (figure 25).

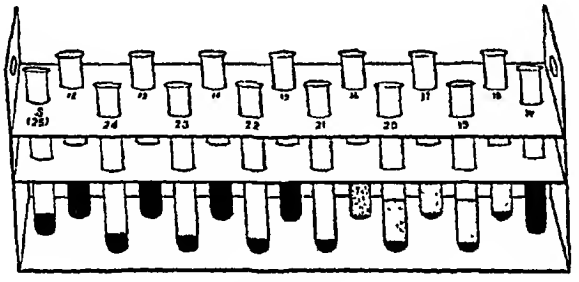

Fig. 25.

Technique

With a syringe fitted with a needle of fairly large bore, or with a capillary pipette, put into each tube the number of drops of 0·5 per cent sodium chloride solution indicated by the number on the rack opposite that tube, and 25 drops in the tube marked S.

Wash out the syringe, or the pipette, thoroughly with distilled water. With the same needle or pipette, add a sufficient number of drops of distilled water to bring the total number of drops of fluid in each tube up to 25, and 25 drops of distilled water in the tube at the end of the rack marked W.

Example.—

Numbers on racks opposite test-tubes.	W	12	13	14	15	16	17	..	..	24	S
Number of drops of 0·5 per cent NaCl to be put into each test-tube.	0	12	13	14	15	16	17	..	..	24	25
Number of drops of distilled water to be added to each test-tube.	25	13	12	11	10	9	8	..	..	1	0

Shake each tube thoroughly to get a uniform mixture. To each tube add one drop, or two drops if the patient is very anæmic, of blood with a syringe, either directly from the patient or from the oxalated blood in the flask. (If great accuracy is aimed at, the test should be performed with washed red cells, as the red cell is protected from hæmolysis by its own plasma.)

Shake all the tubes again. Place the rack with the tubes on a flat even surface and allow it to stand for about 2 hours at room temperature. If there is any special need for hurry, centrifuge all the tubes for about 10 minutes and take the readings at once. If possible, put up a parallel test with normal blood as a check to the accuracy of the sodium chloride solution.

Reading the results.—Hold the rack with the tubes level with your eyes against daylight. Note the number of the tube in which hæmolysis is first shown and also the number of the tube in which it is quite complete. Examine the tubes commencing from tube No. 25 and proceed towards the other end; the first tube showing a tinge of reddish colour in the supernatant fluid indicates the beginning of hæmolysis and the first tube in which all the corpuscles are laked and no corpuscular residue is visible, even after centrifugalization, indicates the point of complete hæmolysis.

The result is expressed as a percentage of sodium chloride solution and is calculated by multiplying the figures on the tubes by 0·02.

Example.—The first tube in which the supernatant fluid shows any pink colour is numbered 20.
The first tube in which there is no corpuscular residue is numbered 15.

Therefore, hæmolysis begins at $20 \times 0{\cdot}02 = 0{\cdot}40$ per cent saline.
hæmolysis is complete at $15 \times 0{\cdot}02 = 0{\cdot}30$ per cent saline.

When the test has to be carried out with very fragile cells, e.g. in acholuric jaundice, concentration of sodium chloride of over 0·5 per cent may be required; in such a case use 1 per cent solution instead of 0·5 per cent and proceed with the test in the same way as before. Take the readings and multiply the numbers on the tubes by 0·04 to get the percentage of the saline.

Normally partial hæmolysis begins at about 0·44 per cent, and is complete at about 0·30 per cent saline.

Quantitative method

The 'qualitative' method would be quite accurate if all the cells in any particular sample were of the same fragility, but the individual cells vary in their fragility just as they vary in their size. It is therefore important to know what percentage of cells have been hæmolysed by each solution. This quantitative method is more laborious, but is worth doing in special cases.

Principle.—A number of accurate red cell counts are done in the usual way except that instead of the standard diluting fluid different strengths of salt solutions* are used; from the results an accurate quantitative graph of the fragility is plotted.

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* Simmel (1923) used mixtures of salts identical with those in the blood, but Whitby and Hynes (1935) obtained identical results by using sodium chloride solutions; we recommend the latter method on account of its simplicity, but the pH should be above 7·0.

Apparatus required

As in red cell count (p. 44).

Also, accurate solution of sodium chloride (standardized against silver nitrate solution) from 0·3 to 0·7 per cent with intervals of 0·05 per cent.

Technique.—Fill red cell pipettes as in the red cell count using different strengths of salt solution ranging from 0·3 to 0·7 per cent with intervals of 0·05 per cent. Allow the pipette to stand on an even surface for about 15 minutes, shake it well, and then count the red cells in the usual way (*see* enumeration of red cells, p. 43 *et seq.*).

Repeat this count with each solution and include a control with the usual diluent.

From the above counts calculate the number of cells that become hæmolysed in different strengths of the salt solution and plot a curve. The normal limit of fragility of the red blood cells, as determined by the quantitative method of Whitby and Hynes (1935), is shown in figure 26.

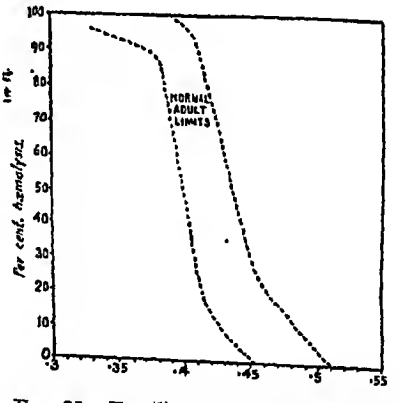

FIG. 26.—Fragility of red corpuscles as determined by quantitative method (Whitby and Hynes, 1935).

Discussion

The fragility test is based on the assumption that there is some parallelism between the fragility of the erythrocytes *in vitro* and their susceptibility to hæmolysis *in vivo*. Though it is very unlikely that the mechanisms of these two processes, neither of which is fully understood, are identical, there is the evidence that in certain conditions, e.g. acholuric jaundice, where the red cells are particularly liable to hæmolysis *in vivo*, they are hæmolysed by hypotonic saline solutions that would not affect normal erythrocytes.

In the physiological process of red cell demolition, there is some evidence that the red cell, normally a biconcave disc, becomes more spherical in shape before it disintegrates, and in many 'hæmolytic' anæmias it has been shown that the red cells are more spherical than the normal red cells. These spherical cells appear to be more susceptible to hæmolysis by hypotonic salt solutions, but it is certain that this is not the only change that brings about this increased fragility.

In all cases of obscure anæmia it is certainly worth carrying out the fragility test, by the quantitative method if this is feasible, not only in the interests of accurate diagnosis but in order to add to our knowledge of the ætiology of the many types of anæmia about which our knowledge is incomplete.

17

Procedures employed in investigating haemorrhagic diseases

Introduction.—In the study of hæmorrhagic diseases and hæmorrhagic diatheses, a number of procedures are commonly carried out that should be included in this series on hæmatological technique. These are :—

A. Estimation of the coagulation time.

B. Estimation of the bleeding time.

C. Quantitative estimation of prothrombin in the blood, or determination of prothrombin time.

D. Capillary resistance test.

E. Study of clot retraction.

F. Platelet count (*vide* p. 61 *et seq.*).

The study of clot retraction gives little additional information of value, as it appears to be dependent entirely on the platelet count, but, as it provides confirmatory evidence, we have included it. The estimation of blood calcium also has some bearing on this subject, but is essentially a biochemical test and will not be described here; further, though calcium deficiency could theoretically lead to a hæmorrhagic state. in actual practice it does not appear to do so, unless associated with other deficiencies.

Discussion.—Briefly stated, and essentially, coagulation of the blood is dependent on fibrinogen, ionized calcium, prothrombin and thrombokinase (thromboplastin). The first three are present in the plasma, while thrombokinase is derived from the disintegration of the platelets and damaged tissue; the chain of events is as follows :—

Prothrombin + ionized calcium + thrombokinase (thromboplastin) ⟶ thrombin.

Thrombin + fibrinogen ⟶ fibrin.

Fibrin + cellular elements of blood ⟶ blood clot.

Clot + platelets ⟶ the retracted clot.

(This leaves out of account the interaction between anti-prothrombin, prothrombin, and thromboplastin substances. Heparin—anti-prothrombin—is a very real substance, but whether it exists in the circulating blood in health does not seem definitely established, so, for the sake of brevity and, we hope, clarity, we have not considered the part it may possibly play in preventing clotting in the circulating blood.)

All the above substances are normally present in the blood; a defect in any of them will cause a break in the continuity of the chain and will lead to hæmorrhage.

Known causes of the hæmorrhagic state are :—

(*i*) Deficiency of platelets (and consequent deficiency of thrombokinase), as in thrombocytopenic purpura.

(*ii*) A defect of the platelets so that they do not disintegrate at the usual rate, as in hæmophilia.

(*iii*) A deficiency of prothrombin in the blood, as in vitamin-K deficiency.

(*iv*) Fibrinogen deficiency, as in prolonged chronic hæmorrhage.

(*v*) A defect in the capillary walls, as in vitamin-C deficiency.

In (*i*) there is a decrease in the platelet count, a normal coagulation, but a prolonged bleeding time, and poor clot retraction ; the prothrombin is normal.

In (*ii*) the bleeding time is normal and the coagulation time is prolonged.

In (*iii*) the bleeding time is normal and coagulation time prolonged ; prothrombin is decreased, but the clot retracts normally.

In (*iv*) the bleeding time is normal and coagulation time prolonged ; and the clot retraction is defective, but prothrombin is normal.

In (*v*) the bleeding time is prolonged and the ' capillary resistance ' is decreased, but the coagulation time, prothrombin and clot retraction are normal.

A. COAGULATION TIME

Various methods have been described for the determination of the coagulation time, but it is essential to adopt one method and to follow the technique as uniformly as possible, for the normal coagulation time varies very considerably with the technique, with the temperature, and with the diameter of the tube used ; it is also influenced by admixture of the blood with tissue juice or any other foreign substances. It is advisable first to determine the coagulation time of a few normal persons in each laboratory ; all subsequent examinations must be done exactly in the same way and the results interpreted in relation to the normal findings.

Two methods are described, as we have had considerable experience with each :—

I. Capillary tube (Wright's) method

Apparatus and reagents required

(*i*) Fine capillary tubes, with diameters from 0·8 to 1·2 mm. and six inches long.
(*ii*) Pricking needle or blood gun.
(*iii*) *Water-bath, or warm water in a beaker at 37°C., with a thermometer.
(*iv*) Spirit lamp.
(*v*) Stop watch.
(*vi*) Alcohol.
(*vii*) Ether.
(*viii*) Cotton-wool.

* This is unnecessary in the hot weather months in India.

Technique.—Clean a finger or an ear lobe, first with alcohol and then with ether. Puncture sharply and fairly deeply with a blood gun or a pricking needle, so that blood will flow freely without the necessity of squeezing. Discard the first two drops of blood, and then fill a capillary tube up to three-fourths of its total length with blood (the capillary tube will fill by capillary attraction when held against the drop of blood). Note the time by the stop watch when the blood first enters the capillary tube. Seal the free ends of the tube in a flame and put it in warm water (37°C.) in a beaker. Repeat the process with two other tubes, noting the time of the commencement in each case.

After one minute, take out the first tube, gently break off a portion of the tube from one end, and repeat this every 15 seconds until a thin line of unbroken coagulum is seen stretched between the two broken ends; note the time. The difference between the two times by the stop watch is the coagulation time. Repeat the procedure with the other two tubes, breaking them at intervals of 15 seconds, and find out the coagulation time for each tube.

Take the mean of the three readings as the coagulation time.

Normal time limits = 1 to 3 minutes.

II. Method of Lee and White (modified) with venous blood

Apparatus and reagents required (additional).

(*i*) An all-glass 5 c.cm. syringe, with a needle of fairly large bore.

(*ii*) Small paraffined test-tubes* ½ inch in diameter with rubber corks, in a rack.

(*iii*) Normal saline.

(*iv*) Hard paraffin in a metal or porcelain bowl.

Prepare a water-bath at 37°C.; place the paraffined tubes ready in the bath in an upright position.

Collect 5 c.cm. of blood from a vein in an all-glass syringe immediately after washing it out with normal saline. Note the time by the stop watch when the blood enters the syringe.

Take out the needle from the syringe, and put exactly 1 c.cm. of blood into each of the three tubes. Cork the tubes with rubber corks and allow them to stand in the water-bath. Wait for 3 minutes, and then every 15 seconds in rotation take a tube and tilt the contents to see if the blood has set. Continue doing this until the blood is found to have set, so that the tube can be inverted without spilling any blood; note the time.

Normal time limits = 3 to 6 minutes.

---

* *Preparing paraffin tubes.*—Melt some paraffin in a clean vessel. Place a test-tube with a glass funnel, which has been previously warmed, in a rack. Pour the melted paraffin through the funnel into the tube up to about two-thirds of its length. Remove the funnel and pour back the excess paraffin from the tube into the vessel. Immerse the tube in cold water, taking care that no water enters the tube; when the test-tube is cold there will be a thin coating of paraffin inside the tube.

Comment.—The second method is more accurate, as certain disturbing factors, e.g. tissue juice, are eliminated.

B. BLEEDING TIME

I. Duke's method

Apparatus required

(*i*) Pricking needle or blood gun.
(*ii*) Filter-paper.
(*iii*) Stop watch.
(*iv*) Sphygmomanometer.

Clean the ear lobe or the dorsal surface of a finger, first with alcohol and then with ether. (The tip of the finger is not suitable for this test as it is horny in many cases.) Prick the selected site fairly deeply with a blood gun or a pricking needle, so that blood flows freely without squeezing. Soak up the blood which comes out with a clean piece of filter-paper without touching the skin. The flow of the blood should be such that the first impression on the filter-paper has a diameter of about half an inch. Without squeezing or pressing the finger, continue to soak up the blood with the filter-paper every 30 seconds until the bleeding stops.

The time from the first appearance of the blood until the cessation of bleeding is the bleeding time.

Normal limits = 1 to 3 minutes.

II. Venous pressure method (Ivy *et al.*, 1935)

Put the cuff of a sphygmomanometer around the arm above the elbow as is done in taking blood pressure. Select a portion of the forearm below the elbow and clean it with alcohol and ether. Raise the pressure and maintain it at 40 mm. of mercury, which is sufficient to check any venous return. Prick sharply up to a depth of about 1/10th inch, at a point free from any superficial veins, so that blood will flow freely. Soak up the drop of blood every 30 seconds, as in method I, until the bleeding stops.

This method is superior to method I, as the capillaries are kept wide open and their tone eliminated; also the area selected for puncture is uniformly thin. For these reasons more consistent results are obtained with this method than with method I.

Normal limits = 2 to 4 minutes.

C. QUANTITATIVE DETERMINATION OF PROTHROMBIN IN BLOOD; PROTHROMBIN TIME

Apparatus required

(*i*) Ten c.cm. record syringe.
(*ii*) Centrifuge tubes.
(*iii*) Small test-tubes in a rack.
(*iv*) Water-bath with a thermometer.
(*v*) Capillary pipettes with teats.
(*vi*) Stop watch.

Solutions required

(*i*) Sodium oxalate solution: dissolve 1·34 of chemically-pure anhydrous sodium oxalate in 100 c.cm. of distilled water.

(*ii*) Calcium chloride solution: dissolve 0·275 of chemically-pure anhydrous calcium chloride in 100 c.cm. of distilled water. Check the strength of the solution by titration against silver nitrate solution.

(*iii*) Thromboplastin solution.

Preparation of thromboplastin solution

Satisfactory results will be obtained with either of the following substances:—

(*a*) From rabbit's brain: Dissolve 0·3 of dehydrated* rabbit's brain in 5 c.cm. of normal saline containing 0·1 c.cm. of sodium oxalate solution, incubate at 45°C. for 10 minutes and finally centrifuge at a low speed for 5 minutes. Pipette off the milky supernatant fluid and keep the solution in a cold incubator, floating a little toluene on the top of the solution. The solution will keep for more than a month.

(*b*) From Russell's viper venom.

A 1 in 20,000 solution of Russell's viper venom gives a potent thromboplastic solution. In the dried state the venom maintains its full potency indefinitely; kept in a refrigerator with toluene, the solution also retains full potency for more than a month (Iyengar *et al.*, 1941).

Technique.—A slightly modified Quick's method is followed:—

Put 0·5 c.cm. of sodium oxalate solution into a centrifuge tube.

Collect blood in a dry syringe (*vide* p. 36) and put 4·5 c.cm. into the centrifuge tube containing oxalate solution; mix well.

Centrifuge until the plasma completely separates; carefully pipette off the supernatant plasma and put it into another dry sterilized tube. Put exactly 0·2 c.cm. of plasma into each of the three tubes marked A, B and C and place them in the water-bath at 37°C.

Take out one tube at a time and to each add exactly 0·2 c.cm. of thromboplastin solution and 0·2 c.cm. of calcium chloride solution.

Note the time when the calcium chloride solution is added; shake gently keeping the tubes in the water-bath; and again note the time when the clot first appears.

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* Dehydrating the brain: Remove all the blood vessels and then macerate well in a glass mortar. Add acetone and macerate again. Decant off the acetone, add some fresh acetone, macerate, and again decant off the excess of acetone. Continue doing this until the whole thing is converted into granular powder; keep in a cold incubator. In the dried state its activity is retained for a long time.

Repeat the experiments with the other two tubes. Take the means of the three headings; this gives the prothrombin time.

Normal = 15 to 25 seconds.

[More recently, we have determined the prothrombin time in a few cases by using Russell-viper venom alone and with ovolecithin added to it in the proportion recommended by Witts and Hobson (1940). With other workers, we have found that the addition of ovolecithin shortens the coagulation time and the results are more uniform.

Normal prothrombin time with ovolecithin = 10 to 15 seconds.]

D. HESS' CAPILLARY RESISTANCE TEST.

Apparatus required

(*i*) Sphygmomanometer.
(*ii*) Stethoscope.
(*iii*) Hand lens.
(*iv*) Skin marking pencil.

Place the cuff of a sphygmomanometer on the arm above the elbow as in taking the blood pressure.

Determine the systolic and diastolic pressures; release the pressure. With a skin (or glass) pencil mark any purpuric spots that may be present on the forearm.

Raise the pressure to a point midway between the systolic and the diastolic and maintain it at this point for 5 minutes. Remove the armlet and examine the forearm in a strong light, and if necessary with a hand lens, for purpuric spots (other than those already marked). A positive result, indicating decreased capillary resistance, is shown by the presence of a number of small fresh purpuric spots distributed over the forearm below the point at which the armlet was applied.

The process should be repeated on the other arm.

E. CLOT RETRACTION STUDY

When blood coagulates, a clot is formed which after a time contracts and expresses the serum; the clot itself then shrinks and becomes hard. Retraction of the clot, as it is called, varies directly with the number of platelets in the circulation but is independent of the coagulation time; with a low platelet count, 60,000 per c.mm. or below, it takes over 24 hours for the clot to separate and the clot is soft and friable.

Apparatus required

(*i*) Same as for collecting blood.
(*ii*) Graduated 10 c.cm. centrifuge tubes, each fitted with a cork having a hole in the centre.
(*iii*) Glass rods with flanges, about the size of a silver 4-anna piece half an inch from the end.
(*iv*) Test-tube rack.

Method of Macfarlane:—Collect blood from the patient in a dry syringe (*vide* p. 36).

Put 5 c.cm. of blood into the centrifuge tube. Put in the cork with the glass rod passed through the hole in the centre of the cork. See that the flanged end of the rod is near the bottom of the centrifuge tube (figure 27).

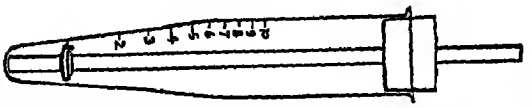

Fig. 27.—Showing centrifuge tube with glass rod in position.

Place the tube in an upright position in the rack and put the rack into an incubator at 37°C.

From one hour onwards, examine the tube from time to time for clotting.

After one hour of clotting, remove the clot by drawing out the glass rod (the clot will adhere to the flanged end of the rod).

Note the amount of fluid in the tube.

The amount of fluid in the tube divided by the amount of blood put in the tube and multiplied by 100 gives the percentage of retraction.

Example.—Suppose 5 c.cm. of blood was taken and 3 c.cm. of fluid was left after the removal of the clot.

Retraction $= \frac{3}{5} \times 100 = 60$ per cent.

Normal retraction = 48 to 64 per cent.

The clot is also examined for firmness and friability.

Clot retraction is low in thrombocytopenic purpura, but is normal in hæmophilia.

18

Red cell diameters : Price-Jones curve

Introduction.—In the normal blood. the red cells are not all exactly the same size, but in no instance does the cell size deviate very much from the mean. However, in some forms of anæmia the mean size of the red cells is much below the 'normal' mean ; in others, it is far above it. But, in the latter case, there are nearly always some exceptionally small cells as well, so that the picture presented is that of cells of a great range of different sizes. This blood picture is best presented in the form of a curve, the abscissa being the size and the ordinates the number of times that cells of the particular size have been encountered (i.e. the frequency). In the normal count, the curve rises sharply from the base line at about 6·0μ to a peak at 7·25μ and then falls almost to the base line again at 8·50μ. This graph is usually known as a Price-Jones curve. In the normal individual the shape of the curve is more constant than the mean diameter of the cells, which in a group of individuals will vary within, but rarely fall outside, the range 6·7μ to 7·8μ.

Price-Jones calculated what he called 'ideal' maximum and minimum normal curves (Price-Jones, 1933).

In different forms of anæmia this curve will vary in shape, its median will move to the right or to the left, and it may be of the normal height, or flatter than normal.

These variations in the curve can be expressed in figures. The median can be given in terms of microns, but it is more usual to give the mean corpuscular diameter (MCD) also in microns; the flatness of the curve is shown by the standard deviation (σ) of the diameters of the cells from the mean diameter, or more accurately by the co-efficient of variation (v), and also by the percentage microcytosis and macrocytosis. These data can all be calculated from the Price-Jones curve.

The measurement of cell volume percentage and calculation of the mean corpuscular volume (MCV) has been described already in an earlier paper in this series; this MCV gives the volume of the cells but not their diameter. Further, it is a 'mean' measurement and from it one gets no idea of the range of size of the individual cells; it is therefore more limited in its application than the Price-Jones curve.

Neither measurement, however, alone will give information on the thickness of the cells, but this can be calculated from the MCV and MCD. The method of making these calculations will be described later.

The technique of the measurement of cell diameters.—The technique described below is a modification of Hynes and Martin's method of measurement of red cell diameters.

The images of the cells are projected on the vertical ground-glass screen of a Bausch and Lomb euscope, at a magnification of 2,000, and the measurements are made with the help of a celluloid protractor on which a series of circles, with diameters increasing by the equivalent of 0·25μ, have been drawn.

Apparatus required

(*i*) A Bausch and Lomb euscope with projection screen.
(*ii*) A mechanical-feed arc lamp with condenser, working at 4·5 amperes.
(*iii*) A glass container filled with distilled water for cooling the beam of light from the arc lamp (filter).
(*iv*) A microscope with oil-immersion objective and eye-piece to give a magnification of 2,000 on the projection screen of the euscope.
(*v*) A stage micrometer scale (Zeiss) with divisions 10μ apart.
(*vi*) A celluloid om./mm. scale.
(*vii*) Celluloid protractor for measuring the cells (*v.i.*).

Preparing the celluloid protractor.—At a magnification of 2,000, 1μ corresponds to 2 mm. and 0·25 to 0·5 mm. The circles on the protractor are drawn with diameters increasing by the equivalent of 0·25μ (0·5 mm.), i.e. the radii of the circles increase by 0·25 mm. The measurements of the radii of the series of circles are best obtained from a diagonal scale drawn to give measurements in multiples of 0·25 mm.

The circles are drawn in Indian ink with very fine bow-pen compasses on a celluloid sheet, with radii increasing from 4 mm. to 12 mm. by steps of 0·25 mm., so as to give measurements corresponding to 4μ to 12μ diameter. Circles smaller and larger than the above are drawn on another sheet. The first sheet is generally sufficient for routine work; the second is needed very exceptionally (*see* figure 28).

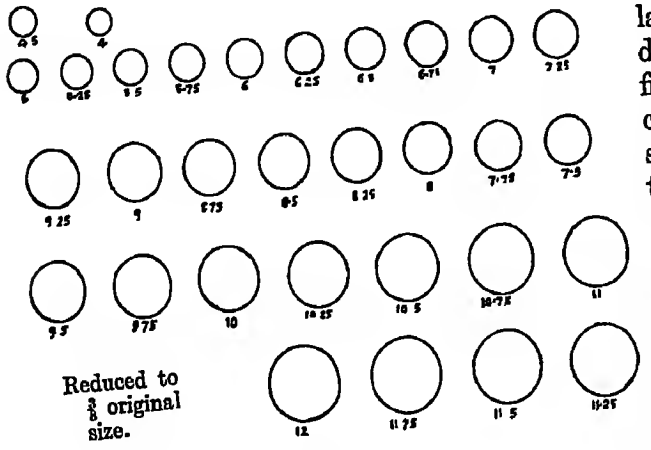

FIG. 28.

Assembling and adjusting the apparatus.—Figure 29 shows the euscope with microscope and arc lamp assembled. The concentrated beam of light from the arc lamp, cooled by passage through water in the filter, is focused on to the *concave* mirror of the microscope which reflects it through the condenser, the objective, and the eye-piece of the microscope. The total reflexion prism of the euscope placed over the ocular reflects the light on to the opaque screen of the euscope. The condenser is fully raised up, and, with the high power objective turned on, a bright beam of light is focused on the opaque screen. After the illumination has been adjusted the micrometer scale is placed under the objective of the microscope and the image of the scale is focused on the opaque screen, first with the low power and finally the oil-immersion objective. A final adjustment of light is made to give the maximum illumination. The opaque screen is now moved off and the image is focused on to the ground-glass projection screen of the euscope. By moving the microscope and/or the prism, the image of the scale is so projected that the lines of the scale are vertical, parallel and entirely free from spherical aberration, at least within an area of about 4 inches by 3 inches marked out around the centre of the viewing screen.

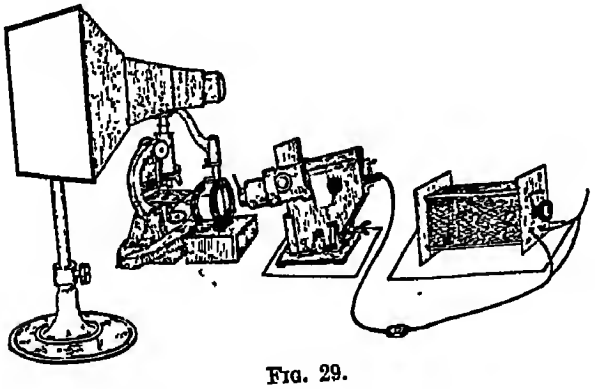

FIG. 29.

That there is no spherical aberration within this area can be shown by noting that the distances between the images of the lines are the same in all parts of this

area. The magnification of the apparatus is adjusted to be 2,000 by using a suitable oil-immersion objective and eye-piece, and varying the length of the draw-tube of the microscope. [A Zeiss microscope with objective 90, and a Leitz eye-piece 15 ×, with a draw-tube length of 154 mm. gives a magnification of 2,000 on the projection screen of the particular euscope used in our laboratory.]

To find the magnification the distance between the images of two lines of the scale is measured with the celluloid cm./mm. scale and, at a magnification of 2,000, the images of the lines of the scale are 2 cm. apart (2 cm. = 20,000μ). [If the different components of the apparatus are kept fixed after the above adjustments, the measurements can be carried out without repeating these adjustments every time.]

Measuring the cells.—The micrometer scale is next removed, the blood film, stained with a Romanowsky stain and counter-stained with 1 per cent aqueous eosin solution, is placed under the oil-immersion objective, and the image of the cells is focused on the ground-glass screen of the euscope. Only those images of the cells that fall within the reduced field are measured. The celluloid protractor with the series of graduated circles is superimposed on the image of the corpuscles to find the circle that fits the image of each cell. Each cell is measured to the nearest 0·25μ. The measurement of circular corpuscles is quite easy. In the case of the irregular-shaped corpuscles, a circle is found such that the area of the corpuscle falling outside the circle is about the same as the area of the circle unfilled by the image of the corpuscle (*see* figure 30). Five hundred cells are measured from different parts of the blood film. It is better to avoid the 'tail' end and the thick parts of the smear, as in the former the cells are excessively spread out and in the latter the cells overlap and are not well spread.

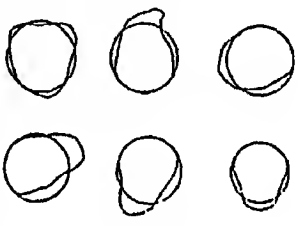

Fig. 30.

Recording the results.—By this method, 500 cells can be measured in about 40 to 90 minutes, the time depending on the size of the cells and the degree of poikilocytosis; the larger and more irregular the cells the longer is the time required. An assistant is needed to note down the measurements and record the number of cells counted with a counting machine of the type of a Veeder counter.

From the figures obtained, the mean diameter, standard deviation, and coefficient of variation are calculated. A Price-Jones curve is drawn by plotting the figures on graph paper. The degree of microcytosis and macrocytosis can also be determined from this curve, by comparison with the maximum and minimum ideal curves; the overlapping of a curve beyond the minimum ideal curve on the low side and the maximum curve on the high side indicates the degree of microcytosis and macrocytosis, respectively.

Method of calculating.—The following figures show the method of calculating the mean cell diameter (MCD), the standard deviation (σ), and the co-efficient of variation (v).

6·5μ, the median, has been taken as the arbitrary mean.

TABLE XVI

Diameters in microns	Number of corpuscles in each class (f)	Deviation (d) of each class from arb. mean, in 0·25μ, i.e. the class interval	f × d		f × d^2
5·25	4	− 5	− 20		100
5·50	18	− 4	− 72		288
5·75	34	− 3	− 102		306
6·00	67	− 2	− 134		268
6·25	74	− 1	− 74		74
6·50	107	0		− 402	0
6·75	70	+ 1	+ 70		70
7·00	50	+ 2	+ 100		200
7·25	46	+ 3	+ 138		414
7·50	18	+ 4	+ 72		288
7·75	5	+ 5	+ 25		125
8·00	4	+ 6	+ 24		144
8·25	2	+ 7	+ 14		98
8·50	1	+ 8	+ 8		64
				+ 451	
	500			+ 49	2,439

n = total number of observations = sum of frequencies (f) = 500.

$\Sigma f d$ = sum (Σ) of the product of the frequencies (f) and the degrees of deviation (d) from the mean = + 49.

$\Sigma f d^2$ = sum of the product of the frequencies (f) and the squares of the deviations (d^2).

Class interval = 0·25μ.

Mean corpuscular diameter (MCD) = arbitrary mean + $\left(\frac{\Sigma f d}{n} \times \text{class interval}\right)$

$= 6{\cdot}5 + \left(\frac{49}{500} \times 0{\cdot}25\right)\mu = 6{\cdot}5245\mu$.

Standard deviation (σ) = $\sqrt{\frac{\Sigma f d^2}{n} - \left(\frac{\Sigma f d}{n}\right)^2} \times$ class interval = $\sqrt{\frac{2439}{500} - \left(\frac{49}{500}\right)^2}$

$\times 0{\cdot}25\mu = \sqrt{4{\cdot}878 - (0{\cdot}098)^2} \times 0{\cdot}25\mu = \sqrt{4{\cdot}878 - 0{\cdot}0096} \times 0{\cdot}25\mu = \sqrt{4{\cdot}868} \times 0{\cdot}25\mu$

$= 2{\cdot}206 \times 0{\cdot}25\mu = 0{\cdot}551\mu$.

Co-efficient of variation (v) = $\frac{\sigma \times 100}{\text{MCD}} = \frac{0{\cdot}551 \times 100}{6{\cdot}525} = 8{\cdot}4$ per cent.

From the mean corpuscular diameter (MCD) and the mean corpuscular volume (*vide* p. 65), the mean corpuscular average thickness (MCAT) can be calculated, by means of the following formula :—

$$\text{MCAT} = \frac{\text{MCV}}{\pi r^2}\mu = \frac{\text{MCV}}{\pi\left(\frac{\text{MCD}}{2}\right)^2}\mu.$$

Example.—Let 90 cu. μ be the mean corpuscular volume (MCV) and 6·52μ the mean corpuscular diameter (MCD).

$$\text{Mean corpuscular average thickness (MCAT)} = \frac{90}{\pi\left(\frac{6{\cdot}52}{2}\right)^2}\mu$$

$$= \frac{90}{3{\cdot}14 \times (3{\cdot}26)^2}\mu = 2{\cdot}69\mu.$$

The normal range of MCAT is from 1·7 to 2·5μ (Price-Jones, Vaughan and Goddard, quoted by Whitby and Britton, 1939).

In order to calculate the percentages of microcytosis and macrocytosis, it is necessary to have 'ideal' minimum and maximum curves for the population in which the investigations are being carried out. Price-Jones calculated the 'ideal curves' for subjects in Great Britain. Working on similar lines, we (Napier, Sen Gupta and Chandra Sekar, 1941) have worked out two similar curves. Two smoothed curves are shown in figure 31 and the expected frequency distributions in the ideal minimum and maximum curves are given in table XVII.

To arrive at the percentage microcytosis and macrocytosis, the number of cells of each size (below 6·9μ), in excess of the number of that particular size that appear in the minimum ideal curve, are summed; this number divided by 5, to reduce it to a percentage, is the percentage microcytosis. Similarly, the number of cells of each size (above 7·8μ), in excess of the number of that particular size that appear in the maximum ideal curve, are summed and divided by 5 to obtain the percentage macrocytosis (*vide* table XVII).

Normals.

	Number of observations.	Mean corpuscular diameter (MCD)μ			Standard deviation (σ)μ			Co-efficient of variation (v) %		
		Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean
London (Price-Jones, 1933).	100	7·718	6·686	7·202	0·500	0·400	0·487	7·30	5·30	6·326
Calcutta (Napier, Sen Gupta and Chandra Sekar, 1941).	25	7·750	6·937	7·344	0·643	0·341	0·492	8·74	4·66	6·697

TABLE XVII

Measurement to nearest 0·25μ	Frequency distributions for ideal minimum curve, MCD = 6·937μ, σ = 0·4948μ	Frequency distributions for ideal maximum curve, MCD = 7·750μ, σ = 0·4948μ	Case X Count	Case X Excess of microcytes	Case X Excess of macrocytes	Case Y Count	Case Y Excess of microcytes	Case Y Excess of macrocytes
4·00	..	..	..	..	..	7	7	..
4·25	..	..	..	..	..	2	2	..
4·50	..	..	1	1	..	7	7	..
4·75	..	..	0	..	..	8	8	..
5·00	..	..	1	1	..	22	22	..
5·25	..	..	1	1	..	30	30	..
5·50	2	..	1	..	..	43	41	..
5·75	6	..	6	..	..	38	32	..
6·00	17	1	3	..	..	56	39	..
6·25	39	4	11	..	..	45	6	..
6·50	68	14	12	..	..	39	..	..
6·75	93	32	29	..	..	41	..	..
7·00	99	61	41	..	..	31	..	..
7·25	82	88	70	..	..	47	..	..
7·50	53	100	50	..	..	27	..	..
7·75	27	88	40	..	..	9	..	..
8·00	10	61	70	..	..	11	..	..
8·25	3	32	40	..	..	9	..	..
8·50	1	14	38	..	6	7	..	..
8·75	..	4	35	..	21	10	..	..
9·00	..	1	18	..	14	3	..	..
9·25	..	..	16	..	15	5	..	4
9·50	..	..	8	..	8	3	..	3
9·75	..	..	1	..	1	..	..	..
10·00	..	..	2	..	2	..	..	..
10·25	..	..	1	..	1	..	..	..
10·50	..	..	2	..	2	..	..	..
10·75	..	..	1	..	1	..	..	..
11·00	..	..	0	..	..	..	..	..
11·25	..	..	1	..	..	..	..	..
11·50	..	..	0	..	..	..	..	..
11·75	..	..	0	..	..	..	..	..
12·00	..	..	1	..	1	..	..	..
			500	3	73	500	196	7

Case X.

Percentage of microcytosis $= \frac{3 \times 100}{500} = 0{\cdot}6$ per cent.

,, macrocytosis $= \frac{73 \times 100}{500} = 14{\cdot}6$ per cent.

MCD = 7·791μ.

σ = 0·903μ.

$v = 11{\cdot}5$ per cent.

Case Y.

Percentage of microcytosis $= \frac{194 \times 100}{500} = 38{\cdot}8$ per cent.

,, macrocytosis $= \frac{7 \times 100}{500} = 1{\cdot}4$ per cent.

MCD = 6·434μ.

σ = 1·07μ.

$v = 16{\cdot}6$ per cent.

Significance of the findings.—It is not possible here to enter into any extensive discussion on the significance of the different findings, but as a general rule it may be taken that in cases that have a high microcytosis percentage and a low MCV the anæmia is due to iron deficiency. When such microcytosis is associated with a normal, or even a high MCV, it means that the cells are thicker than

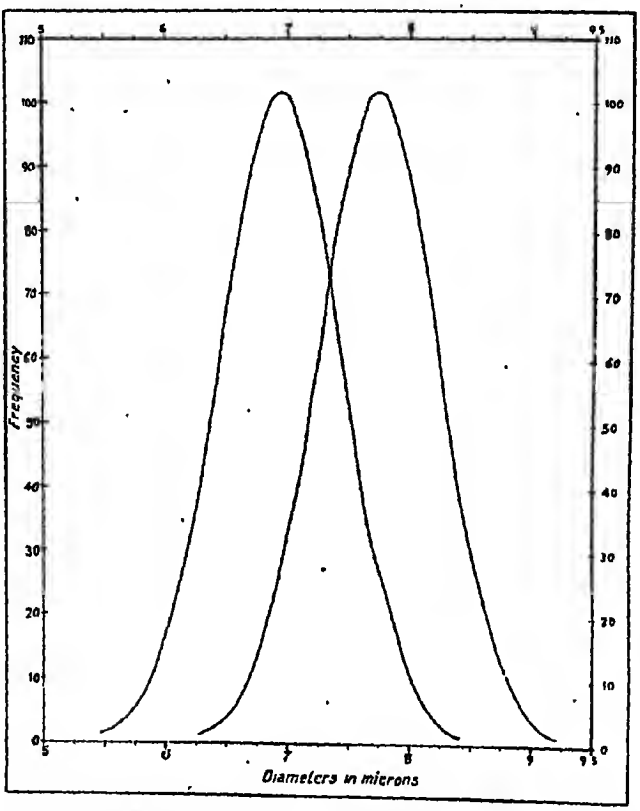

FIG. 31.—Ideal maximum and minimum curves based on Indian data (Napier, Sen Gupta and Chandra Sekar, 1941).

normal and there is a tendency to spherocytosis; such a condition is usually found in hæmolytic anæmias. Pernicious anæmia shows a flat curve with a wide base, usually a high percentage of macrocytes and a few microcytes, whereas tropical macrocytic anæmia is usually associated with a moderately tall curve with an MCD slightly on the macrocytic side, a few macrocytes, and probably no microcytes.

19

The investigation of a case and the reporting of results

The question that we are frequently asked is, what do you consider the minimum examinations that should be carried out in a case of anæmia? We should be forced to reply that, with the exception of those in section 17, which apply particularly in hæmorrhagic diseases, *at least* all the examinations that have been described in this little book should be made for the proper investigation of a patient.

In actual practice, nearly all the blood examinations can be carried out from a single specimen of blood and do not take a good technician more than an hour, if the Price-Jones curve is excluded. A certain amount of time can be saved by omitting the Arneth count, the fragility test, and the enumeration of thrombocytes, and there are many circumstances in which these tests will give little information of practical value. This leaves the hæmoglobin estimation, the total red and white cell counts, the reticulocyte count, cell volume percentage estimation (hæmatocrit) and sedimentation rate, van den Bergh's reaction, and white cell differential count, to be done as a minimum routine procedure in every case.

The findings will indicate whether these examinations should be supplemented. Sternal puncture gives very valuable additional information, but it must not be considered as a short cut to diagnosis for it will seldom be of any value without a complete blood count, and further the identification of the various cells is very skilled work that necessitates considerable experience.

There will be occasions when electric current is not available and in these circumstances it will not be possible to estimate the cell volume percentage satisfactorily. Although the mean corpuscular hæmoglobin (MCH) usually runs more-or-less parallel with the mean corpuscular volume (MCV), it will be advisable to make some measurement of the red cells, and, as in our experience clinical halometers are very inaccurate, the mean cell diameter should be measured by some modification of Price-Jones method.

The inclusion of the erythrocyte sedimentation rate (ESR) in the minimum procedures might be questioned, but, if a Wintrobe's tube is used for cell volume estimation, the ESR requires no additional apparatus and entails no extra labour, except the taking of a reading at the end of an hour, and the information it gives is often useful.

Gastric analysis is important when the question of pernicious anæmia arises and in some cases of microcytic anæmia of doubtful ætiology, but except in these cases and as a general procedure in investigating the function of the gastro-intestinal tract, dysfunction of which is so often an ætiological factor in anæmia in India, we would not give it a high place in the order of importance, in the investigation of cases of anæmia in this country, unless there are associated gastro-intestinal symptoms.

For the complete investigation of the ætiological factors many other examinations will have to be carried out, e.g. the urine for albumin, blood, and ova; the stools for protozoa, helminths, and pathogenic bacteria, for fat content, and for the presence of occult blood; and the blood for 'blood grouping', the Wassermann reaction, cholesterol, albumin/globulin ratio, etc.

Controlling progress.—Circumstances will usually dictate how often blood examinations should be carried out during treatment, but there are certain points which we will consider here.

In the first place, when a seriously ill patient is admitted into hospital, he is often suffering from some degree of hæmo-concentration, and a second examination made a few days after admission may show a marked drop in hæmoglobin percentage, whereas the patient's condition may have improved considerably. If allowance is not made for this fact, the effect of the first treatment that is given may be misjudged, and we have always adopted the practice of making a second examination before prescribing specific treatment (except in urgent cases where an immediate blood transfusion is indicated).

Further, there are many nutritional anæmias in India in which there is a steady improvement directly the patient is put to bed and given a good diet. Allowance must also be made for this fact, if the value of any special form of treatment is being estimated and, in such circumstances, at least two weeks of rest and diet should be allowed before the specific treatment is started.

We make a practice of repeating the blood examination, including hæmoglobin percentage, red cell count, reticulocytes, cell volume percentage, van den Bergh's reaction, and ESR, and examination of the film for abnormal cells, once a week, or earlier if a set-back is suspected or if the treatment is to be changed. In special cases other examinations, such as the platelet and leucocyte counts, are also repeated.

When any specific form of treatment is given a reticulocyte count is done daily from the 4th until about the 12th day, or, in a case where there is a reticulocyte crisis, until the reticulocyte percentage has fallen to its previous level.

The printed forms that we use in the School are shown below. There is quite possibly room for improvement in these forms, and we have modified them from time to time, but we find that, without being cumbersome, they are sufficient for our purpose.

Form 1 is the ordinary report form which is sent to the ward and attached to the notes; size 8½ by 6½ inches.

Form 2 is the sternal puncture report form, similarly sent to the ward and attached to the notes; a résumé of this is entered on the reverse of form 3; size 8½ by 5½ inches.

Form 3 is the card which we keep in the laboratory. The important data on which progress is judged are entered on the face of the card, and other data and records of examination that are not usually repeated, on the reverse; size 8½ by 6½ inches—stiff card.

FORM I

S. T. M. ANÆMIA DEPT.

BLOOD REPORT

Name..... *Sex*..... *Age*..... *Ward*..... *Bed*.....

Hæmoglobin (Helligo normal hæmometer) .. .. .. per cent.

gm. per 100 c.cm.

Red blood cells .. .. per c.mm.

Reticulocytes .. .. per cent.

Cell volume .. .. per cent.

Mean corp. volume .. .. cu. μ.

Mean corp. hæmoglobin .. .. $\gamma\gamma$

Mean corp. hæmoglobin conc. .. .. per cent.

White blood cells .. .. per c.mm.

	per cent.	per c.mm.
Neutrophils		
Lymphocytes		
Large mononuclears		
Eosinophils		
Basophils		

Arneth count, I II III IV V Weighted mean *Blood group.*

Abnormal cells.

Platelets .. .. .. per c.mm.

Coagulation time .. .. *Bleeding time.*

Fragility of red cells .. .. *Prothrombin time.*

Capillary resistance.

Sedimentation rate (1 hour) { Observed / Corrected—

Van den Bergh's test { Direct / Indirect — mg. per 100 c.cm.

Opinion.

Date.....

FORM 2

S. T. M. ANÆMIA DEPT.

STERNAL PUNCTURE REPORT

Name.....*Age*.....*Ward*.....*Bed*.....

Total nucleated cells.....Reticulocytes.....

Endothelial cells.

Red cell series.

Megaloblast.....

Erythroblast.....

Macroblast.....

Normoblast.....

White cell series.

Granular series—

Myeloblast.....

Pro-myelocyte.....

Neutro. myelocyte { A..... B.....

„ meta-myelocyte.....

„ band.....

„ segmented.....

Eosino. myelocyte.....

„ meta-myelocyte.....

„ band.....

„ segmented.....

Basophils.....

Non-granular series—

Lymphoblast.....

Lymphocyte { A..... B.....

Plasma cell.....

Monoblast.....

Pre-monocyte.....

Large mononuclear.....

Megakaryocyte.....

Undifferentiated.....

Parasites.....

Date.....

S. T. M.

FORM 3

ANÆMIA CARD

ANÆMIA DEPT.

Name..... *Sex*..... *Age*..... *Ward*..... *Bed*.....

Date	Treatment and diet	Hb	RBC	Ret	CV	MCV	MCH	MCHC	Total	Leucocytes					v.d.B.	ESR	Abnormal cell
										N	L	M	E	B			

FORM 3 (reverse)

N, AF

Blood :

Platelets | Fragility | Coagn. time | Bleeding time | Cap. res. | Prothrombin time
Arneth count : I II III IV V | Wt. mean | W. R. | Blood group

Sternal puncture :—

Total | Gran | Non-gran | Red | Mgbt | Erbt | Mac | Norme | Ret

Spleen puncture :—

Gastric analysis :— F. $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{4}$, $1\frac{1}{2}$, $1\frac{3}{4}$, 2, $2\frac{1}{4}$, $2\frac{1}{2}$, 3 hours.

Price-Jones curve

Peptic digestion

Mean corpuscular diam. (MCD)
Standard deviation (*s.d.*)
Coefficient variation (*v*)
Per cent macrocytosis
„ „ microcytosis

Urine :

Stool : { Ova / Protozoa / Occult blood

Other data

Spleen :

Clinical notes

Liver :

Weight :

Epidemiological data

Economic status

Dietary habits

Residence—malarious or not

10 Occupation

Family history

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PART III

1

The need for anaemia centres

THE fact that anæmia does not present a major problem in Western Europe, America and Australia suggests that a large proportion of the cases which occur in India are in the light of present knowledge preventable and indeed may be expected to disappear spontaneously when standards of living are higher, epidemics brought under control and the knowledge of treatment of anæmia more widely disseminated.

In India, however, we are faced at present with this problem of anæmia which has come at last to be recognized as important, not only in causing widespread morbidity but also as responsible for a considerable mortality, this mortality being particularly high amongst women submitted to the extra strain of pregnancy. Further, not only is anæmia directly responsible for much chronic poor health and lowered efficiency but it predisposes to other infections, particularly sepsis after child-birth and abortion.

Anæmia, although due largely to other and preventable conditions, cannot be dismissed as something which will right itself as causative factors come under control. The condition needs much further investigation and in its major and minor forms a wider recognition and more adequate treatment.

We find ourselves faced with—

(*a*) lack of knowledge or incomplete knowledge regarding the ætiology of many of the forms of anæmia commonly encountered in India;

(*b*) lack of knowledge regarding the distribution of forms of anæmia in different localities;

(*c*) the need for wider recognition of the problem amongst medical and public-health workers and the general public, especially of the danger of anæmia when it occurs in a severe form associated with pregnancy;

(*d*) the need for special facilities for the hæmatological examinations which are necessary to provide accurate diagnosis of the type of anæmia present on which basis alone treatment can be carried out in a scientific and effective manner;

(*e*) the necessary means of treatment which ought to be made available for those unable to pay the cost, e.g. of drugs, injections, blood transfusion, etc.

It is suggested that the most effective way of meeting these needs is the establishment of anæmia centres or clinics, either in separate buildings or in association with already existing institutions. We have clinics for tuberculosis, leprosy, kala-azar, etc.; why not anæmia clinics? The objection may be raised that anæmia is a symptom occurring in many diseases. This is true, but there are also many forms of 'primary' anæmia, which though they are possibly only 'primary' because we do not yet know their cause, require special investigation before any attempt can be made either to treat or to prevent them. Progress in the knowledge of heart diseases, to take an example about which a similar criticism might be made, has been made through the agency of cardiological departments or institutes for the purpose. Further, because our knowledge is so incomplete regarding many ætiological factors, social, environmental, and dietetic, which are believed to be important in the development of anæmic states, as well as the infections which actually cause anæmia or predispose to its development, special centres which concentrate on all these aspects of the problem should be instituted.

The limited facilities which exist to-day in all but the fewest places make it all but impossible for the practitioner and ill-equipped institutions to treat cases of anæmia scientifically, because the means of investigation are lacking. The result of this is generally that the underlying cause in a particular case of anæmia is not discovered, so that even if thorough treatment is given the condition again recurs. Or again a case of anæmia is treated by too little of the required drug or for too short a time with little effect; at other times either polytherapy or 'hit-or-miss' procedures are adopted which are both wasteful and expensive.

The work of an anæmia centre or clinic should include diagnosis, treatment, and research.

(*a*) *Diagnosis.*—Facilities should be available for hæmatological and other pathological investigation of cases of anæmia sent from institutions or by general practitioners in the neighbourhood. The diagnostic report might include an indication of the line of treatment which is likely to be successful and the date at which another examination should be made.

(*b*) *Treatment.*—Treatment should be provided for those unable or unwilling to obtain it elsewhere. Cases of a mild degree of anæmia can be treated as out-patients; others will need in-patient treatment and the provision of beds in connection with the work of the anæmia clinic is an important question. This will be easily solved if the clinic is attached to a medical or obstetrical department. The cost of an anæmia centre in a separate building will be greatly increased by the provision of beds, and a more practical scheme would be an arrangement for accommodation in one of the existing hospitals. If the centre is to function to its greatest possible extent beds will certainly be required. The treatment of anæmia is often expensive and needs to be given for a prolonged period so that many persons are not able to obtain the necessary treatment unless it is provided free of charge

But at present anti-anæmic drugs are often wasted because treatment is not controlled by hæmatological and other investigations.

(c) *Research.*—The anæmia centre or clinic is the ideal place from which to carry out an anæmia inquiry, and if arrangements are available for providing treatment the work of such an inquiry is greatly facilitated.

The cases sent for diagnosis to the centre will provide a general impression of the distribution of the types of anæmia in the locality. The lines of further investigation likely to be productive will become clear as work proceeds. The treatment clinic will provide mild cases for observation and make it possible to try out various kinds of anti-anæmic drugs. The in-patient beds will provide material for more complete investigation and prolonged observation.

Staff

The officer in charge of the centre should be a physician with special experience in hæmatological work. The problem of anæmia is many-sided and needs to be tackled from the laboratory, the clinic, the bedside, and the field. It is important that the officer in charge should be at home in all these places. The slow progress of knowledge regarding the importance of anæmia as a problem in India may be largely attributed to the fact that many of the most severe cases (associated with pregnancy) are still confined to the homes of the people and are seen only by untrained attendants at child-birth, or are admitted to purdah hospitals where facilities for laboratory diagnosis are often meagre. Frequently, blood examinations are carried out in laboratories where the pathologist does not see the clinical condition or watch the progress of the case and knows still less about the ætiological factors, except in so far as other abnormal pathological findings are concerned. In order that the whole picture of a case of anæmia may be squarely faced, the background, environmental and personal, the diet and family customs, must be recognized as likely factors in the development of the condition and must be given the same consideration as is applied to the blood picture and the clinical findings.

The number of staff of the centre in addition to the officer in charge will depend on the volume of work which is to be undertaken and whether beds are associated. It is suggested that two medical assistants at least will be required, one of whom should have hæmatological experience and the other should be a woman with obstetrical experience. A health visitor for follow-up work in homes will be an essential requirement.

There is something to be said for a pathologist having a detached viewpoint but in the case of anæmia work it is suggested that there is more to be said for making workers share in the three-fold activities of the centre, namely, laboratory work, clinical work and field work.

Publicity

The propaganda value of an anæmia centre should not be forgotten. The mere fact of its existence has a value in itself, but after a few years of work it

should be possible, if the information collected is analysed, to draw conclusions regarding the causes of anæmia in the locality and to indicate a plan of action whereby prevalent forms of anæmia may be prevented.

2

Conduct of an anaemia inquiry

Choice of place or population

The choice of place for an anæmia inquiry will probably have been indicated by hospital or dispensary reports of a high frequency of severe anæmia in the locality, or perhaps particularly in one section of the population.

Before deciding finally to undertake an anæmia inquiry in a particular area, it is suggested that visits should be paid to the institutions for medical relief in the district, particularly women's hospitals and welfare centres, to the health officer and to representative general practitioners. Useful information regarding the incidence of anæmia in the locality, the forms of the disease encountered and prevailing impressions in regard to its ætiology will thus be gained, and confidence will be established between the officer in charge and the local medical and public health workers. It may be possible to obtain figures regarding the incidence of anæmia from some of the institutions visited, and the degree of reliance which can be placed on the causes of death in the vital statistics may become evident in the course of friendly discussions. The contacts made and the information collected should make it possible to find out what line of inquiry will be likely to be most productive and what degree of co-operation will be obtainable.

It is to be expected that the most productive type of inquiry on a small scale will be one in which a number of common factors exist in the environment, general and personal, of the group which is studied and the control group, for when this is the case the ætiological factors are more likely to stand out clearly.

A further point to remember, in the investigation of cases of anæmia in pregnant women, is that the period of time during which the case must be kept under observation is about a year and this will be easier in a controlled population such as is found in a tea garden or in certain industrial areas. Valuable help in keeping track of cases should be obtainable from the staff of welfare centres if health visitors are employed, and to a lesser extent from the pre-natal department of hospitals.

The hæmatological background

It may be possible to obtain sufficient idea of the frequency of blood diseases in the general population from figures obtained from institutions and workers in the locality or from work which has already been done. If this is not so, random samples of blood may be taken for hæmoglobin examination by a rough method (e.g. Tallqvist) in, say, every person attending a hospital or dispensary on a single

day, and again by examining the hæmoglobin by the same method in students or school children in schools in different areas or of different economic status. The most helpful method of inquiry into the background will depend on the group which is chosen for fuller investigation.

The selected group

This may have been already decided upon because of a high incidence of anæmia in a certain group. If not, two main types of cases may be selected for study—

(*a*) from a more-or-less controlled population, e.g. workers in jute or cotton factories, miners, tea-garden coolies, students;

(*b*) from an uncontrolled population, e.g. hospital patients, drawn possibly from a wide area and difficult to follow up, or the welfare centre clientele, a mixed population but more restricted as to the area from which the people come.

Whichever type of group is selected a control group will probably be necessary and it is desirable that the control group should have, as far as possible, factors in common. For example, amongst the controlled population the control group should preferably be the non-anæmic persons doing the same work, or chosen from non-workers living in the same neighbourhood. In the case of the uncontrolled population, it may be possible to get a control group from a hospital in a different locality, for example, suburban as compared with a congested area or from a welfare centre in a different district.

Another series of cases which might be selected for investigation is the population in a given area after a severe epidemic, e.g. of malaria, or after shortage of rain and failure of crops have affected the health of the people.

Period of inquiry

Clinical observation indicates that severe cases of anæmia come under observation more commonly at certain times of the year and also that different forms of anæmia have different seasonal variations. The period of an anæmia inquiry should therefore be at least two years. In the case of pregnant women the period during which a single case must be studied is about a year.

Local epidemic and endemic diseases

Some indication regarding these will be obtainable from local hospitals and public health records before the work begins. But every effort must be made to get more information on the subject as the inquiry goes on, and all workers should be taught the importance of enquiring into and reporting the existence of such diseases whenever they come across them, or when they get a history of them, either in the families concerned or in the neighbourhood. The assessment of the part played by such diseases in the causation of anæmia will be one of the most important conclusions in the report on the inquiry.

The anæmic group and the control group

Anæmic group.—It is suggested that not less than 150 individuals (of which 50 should be men, 50 women, and 50 pregnant women) should be fully investigated.

Control group.—This should consist of at least 100 individuals of which 50 should be pregnant women. To provide these figures as a minimum on which results can be based, considerably more than this number may have to be examined, especially of pregnant women, to make up for unsuitable or incomplete cases. The ages of the individuals in the two groups should be similar and should lie between early adult and middle life. When students or school children are investigated, the control group should consist of individuals of similar ages.

The control and anæmic series can be selected in one of two ways—

(*a*) If the population contains a large number of anæmia cases, estimate the hæmoglobin of each adult in the population and plot the findings in groups of 0·5 gramme on graph paper. The so-called normals will usually fall into a normal curve and the anæmic individuals will be arranged irregularly below. It will then be possible to select two groups, the normals (control) and the anæmic, though the actual point of division may not be clear-cut and an arbitrary figure may have to be taken. Full investigation may then be carried out in a number of individuals of each group.

(*b*) Or, if there are fewer anæmic individuals in the population, the controls may be selected from the apparently fit individuals, and the full investigation carried out. Those that show obviously abnormal features should be excluded from the finally selected normals. Similarly individuals showing hæmoglobin estimation and those that fall below some arbitrary level (*see* below) are selected for complete investigation.

Criterion for anæmia

This must be an arbitrary figure. In a rural population it will not be feasible to consider anything higher than 7 grammes as severe anæmia, but in a city population, the vast majority of women may be above 10 grammes and any figure below this, but above 7 grammes, might be considered as indicating slight anæmia.

In selecting patients as anæmic, it will be as well to consider only one feature of the blood picture, the hæmoglobin level.

Method of work

Control group.—A physical examination, as indicated on the control group (Form B), should be carried out in each case as well as a blood examination. One examination only in the control group is sufficient.

The anæmic group (or cases for full investigation).—Each anæmic individual, man and non-pregnant woman, should have two full examinations of the blood and a physical examination on the lines indicated in the form for anæmics. The interval between the two examinations should be two to three months. A home visit is necessary in each case in order to complete the form and gain the necessary background information.

Pregnant women should be examined at least three times, i.e. twice in pregnancy and once after delivery at the following periods :—

First examination in the second trimester of pregnancy, i.e. between the 12th and 24th week of pregnancy and as nearly as possible at the 20th week. During this period the uterus rises from the level of the symphysis pubis to the upper border of the umbilicus. At the 20th week the upper border of the fundus is at the level of the lower border of the umbilicus in the recumbent position and the mother may be expected to have just begun to feel fœtal movements. This is the time of choice for the first examination, because the minor complications of early pregnancy have passed.

Second examination in the third trimester of pregnancy. The period of choice is between the 32nd and 34th week.

Premature delivery is common in anæmia, and therefore the second examination should not be unduly delayed. In severely anæmic women, it may be better to do it about the 30th week. After the 34th week the later complications of pregnancy are more likely to intrude, and other pregnancy toxæmias and œdema due to pressure may complicate the picture and make the interpretation of findings more difficult. It is suggested that, because of the normal variations in the blood which occur in pregnancy, it is better to try to eliminate the effect of these by examinations in all cases as nearly as possible at the same stages of pregnancy.

Third examination (post-natal)—between the 3rd and 6th month after delivery, the period of choice being the 4th month. Special note should be made as to whether lactation is proceeding or not.

Further examinations will probably be made in addition to the above, but it is suggested that those described are the minimum required and in analysis are those which should be used.

Note.—When a case of anæmia is found associated with such diseases as tuberculosis, malignant disease or chronic nephritis, at the time of the first examination, it is suggested that the case should be included but that further examinations should not be made. These cases are of interest in a series of anæmic cases but are not likely to throw light on the causation of the more obscure anæmias in this country, and therefore it is better not to spend time investigating them if other cases of more doubtful ætiology are available. On the other hand, a case with a history of malaria, especially chronic malaria of very long standing, and of chronic or subacute bowel disease should be fully investigated. The anæmias following

these conditions are by no means clearly defined, and the importance of the part played by tropical infections in the causation of the tropical anæmias is one of the most important points that the inquiry should help to elucidate.

The forms

Form A for anæmia cases (or cases for full investigation).

Form B for the control group.

The forms are mainly self-explanatory. At least one home visit will be required, and the information regarding delivery (in the pregnant cases) will probably require visits to institutions or attendants.

The general nutrition and the diet, quantitative and qualitative, taken by the individual need careful investigation, and association with a nutrition survey is valuable, but this question and that of personal habits (e.g. hours of work, sleep, exercise and the family customs) need to be intelligently considered and not mechanically noted. For example, in a meat-eating family the mother who may be the subject of investigation may herself take practically no meat if she eats what remains after the other members of the family have eaten. Similarly, lack of sunlight entering a particular room where the woman spends 21 hours of the day may be a fact of more importance than the degree of ventilation of the whole house. The workers in an inquiry should be taught to search for information such as this when filling up the forms.

No quantitative estimation of the articles of food should be attempted unless this is provided by a diet survey officer. The difficulty in assessing the importance of diet in causing anæmia lies in the fact that it may be the diet the individual was consuming some weeks or months ago that is important, rather than the food she is eating at the time of inquiry. It is therefore probable that general rather than specific findings may be more helpful.

The greatest difficulty will probably be encountered in obtaining specimens of fæces for examination, but it is urged that this should be managed if possible, at least on one occasion. Failure to possess this information will handicap the report to a very marked degree.

The hæmatological examinations required will be clear from the forms and it should be emphasized again that uniformity of method in the collection of specimens and in pathological technique should be maintained throughout the inquiry. Changes of workers should be avoided as far as possible, and it is most important that the workers employed should have been trained in special methods of hæmatological technique which are similar to those used in similar inquiries in other places, and that the instruments used should have been standardized.

FORM A

ANÆMIA INQUIRY

Serial No..... Hospital, Clinic, etc.....

Date..... Reg. No.....In-patient.....Out-patient.....

Name..... Address.....

Place and Province of origin.....Religion.....Age.....Sex.....

Occupation (state).....(of husband, of woman).....

Home conditions: No. of rooms.....No. of inmates under 12.....over 12 years.....

House clean, fair, dirty. Overcrowding.....Drainage.....

Economic status: poor, middle class or rich. (Remarks.....)

Diet (ordinary of family).....rice, wheat, meat, fish, eggs, green vegetables, fruit.....

Milk (or curds) taken by individual daily.....

If any change in diet lately, state.....

Previous illness (particular inquiries should be made regarding the following: recent loss of blood, malaria, bowel diseases, deficiency diseases).

Physical examination.....symptoms and their duration:—

Presenting symptoms, if any.....weakness.....shortness of breath.....

palpitations.....swelling of feet or body.....sore mouth.....

indigestion.....constipation.....diarrhœa.....others.....

Signs: Nutrition.....Pulse.....Temperature.....Blood pressure.....

Pallor.....lemon tinge.....jaundice.....œdema.....

Nails cracked.....spoon-shaped.....Tongue, pale.....smooth.....flabby.....

red.....sore.....furred.....Heart dilated (state degree).....murmurs.....

Lungs..... Wassermann reaction.....

Abdomen.....spleen.....liver.....ascites.....

Septic foci detected.....

Pathological reports

Date	Home or Inst.	Hb	RBC	Retic	WBC	Cell Vol.	MCV	MCH	MCHC	van d.B	Alb. in urine	Fæces

FORM A—*contd.*

In the case of women the following information should also be obtained.—
If purdahnashin.....(or relatively purdah.....)
Pregnant.....weeks, puerperal.....weeks. Not pregnant:.....
Menstruation.....(state type). Date of last m. p.....

Obstetric history

Number	Term. prem. abortion	Complic. of preg. or labour	Infant		
			Alive or still-born	Healthy	Died, age and cause

Progress of pregnancy

Date	No. of weeks preg. or puerperal	Height of fundus	Blood pressure	Urine	Remarks

Labour: Date of delivery.....place.....attendant.....
normal.....complicated (state).....
Hæmorrhage less than normal, normal, excessive.....placenta delivered spontaneously, or other method of delivery.....
Infant: alive, dead, full-term, premature.....months, abortion.....months
Puerperium normal.....complicated.....fever.....

Remarks by investigator.

FORM B

Serial No.---------- Date----------

Name or number---------- Single—Married—Widow.

Age----------Religion----------

Economic status (poor : middle class : rich) ----------

Past illness----------

Menstrual history—First menstruation----------

Regular—Irregular----------

Scanty—Normal—Profuse----------

Pain----------

Constitutional symptoms----------

Obstetric history—No. of children—Date of last----------

No. of miscarriages—Date of last----------

General diet :—Rice, chapati, dâl, vegetable, fish, meat, egg, fruit, sugar, milk, tea.

Appetite----------Digestion----------Bowels----------

Examination of the patient.

General appearance----------

Anæmia----------Jaundice----------

Teeth----------Tongue----------Gums----------

Pulse rate----------Puffiness of face----------

Heart----------Liver----------Spleen----------

Pitting of tibiæ----------

Hæmoglobin in grammes per 100 c.cm.----------

Red cells in millions per c.mm.----------

Reticulocytes per cent of red cells----------

Cell volume per cent----------

MCV----------

MCH----------

MCHC----------

Leucocytes----------Differential count P. L. M. E.

Blood pressure----------

Van den Bergh (indirect) mgm. per 100 c.cm.

Sedimentation rate----------

Wassermann reaction----------

Urine----------

Fæces----------

APPENDIX I

Summary of the findings, conclusions and recommendations of an anæmia inquiry in Calcutta by L. E. Napier and M. I. Neal Edwards under the Indian Research Fund Association, 1937–39*

Summary of findings

Blood examinations were carried out and an analysis is made of the blood picture in 128 'normal' non-pregnant women, in 64 'normal' pregnant women and in 467 pregnant women clinically chosen as anæmic; of these women of the last group, 386 were first examined before parturition and 81 after parturition.

For the purpose of analysis the anæmic women were divided into three classes, A—markedly anæmic, 50 per cent or less (Hellige), B—moderately anæmic, over 50 per cent and under 68 per cent, and C—68 per cent or over; this last class acted as additional controls. The cases were further divided into three hæmatological groups, microcytic, normocytic and macrocytic, so that there were altogether nine sub-groups. Epidemiological, pathological and clinical data were analysed with reference to these classes, groups and sub-groups. From this analysis the following conclusions were arrived at:—

1. The 'normal' pregnant women show a distinctly lower hæmoglobin level than the normal non-pregnant women (reported elsewhere) but no evidence of the progressive reduction of hæmoglobin percentage throughout pregnancy that has been reported by other workers; there is however evidence of a tendency to increase in cell size as pregnancy progresses.

2. Amongst the anæmic patients the same tendency towards increase in size of the red cell throughout pregnancy is apparent, but otherwise there is no constant change in the size of the red cell; macrocytic and microcytic anæmias usually maintain their own characteristics until cure is effected.

3. There is evidence that the removal of the fœtus, naturally, at term, or prematurely, was a more potent factor than any treatment that may have been given in bringing about improvement in the blood picture, in the macrocytic and normocytic cases, but less so in the microcytic.

4. There is evidence that there is an iron deficiency in the large majority of the cases under investigation. This iron deficiency was most evident in the class that showed the least anæmia, this anæmia-producing factor probably being over-shadowed by other factors in the more anæmic classes, and though it is

* Indian Medical Research Memoir, No. 33, Thacker, Spink & Co. (1933), Ltd., Calcutta, December 1941.

more prominent in the microcytic groups, there is evidence of some degree of iron deficiency in the other groups.

5. Liver injections appeared to be effective in some individual cases, but, in the analysis of the cases as groups, there was no evidence of this effect, perhaps because a negligible number of injections were given in many cases.

6. Macrocytic anæmia is more common amongst primigravidæ; this association is independent of age, for otherwise there is less anæmia amongst young women. Microcytic anæmia is commonest amongst older women.

7. Of the severe cases coming under observation early in pregnancy, the majority were macrocytic.

8. A distinctly larger number of macrocytic cases come under observation during the second half of the year.

9. There is a very marked association between poverty and anæmia, and some correlation between macrocytosis and poverty. In the richer classes, vegetarianism appears to be correlated with macrocytic anæmia.

10. There is a significant correlation between Mohammedanism and microcytic anæmia, and also therefore with purdah.

11. There is a negative association between excessive hæmorrhage at parturition and both ante-partum anæmia and macrocytosis.

12. There is no correlation between sore mouth and the degree of anæmia, but there is a suggestion of association between macrocytosis and sore mouth.

13. There is a significant association between diarrhœa and to a less extent vomiting and macrocytosis.

14. There is a significant association between both severe anæmia and macrocytosis and œdema.

15. There is a significant association between severe macrocytic anæmia and both spleen and liver enlargement.

16. Albumin in the urine is significantly more frequent in the severe anæmia class.

17. Hyperbilirubinæmia is definitely correlated with severe anæmia and macrocytosis.

18. There is a positive correlation between reticulocytosis and both the degree of anæmia and macrocytosis.

19. In the severe-macrocytic-anæmia group hyperbilirubinæmia is correlated significantly with liver enlargement and spleen enlargement, but in the rest of the cases in the severe anæmia group it is correlated only with splenic enlargement.

20. There is considerable evidence of an ætiological association of syphilis, or some other Wassermann positive producing factor, in the severe anæmia groups, for a positive Wassermann reaction was obtained in over 20 per cent and in over 25 per cent of the 'poor' patients of this group; in the case of the latter the difference between the macrocytic groups and the rest is significant. There is

also a strong suggestion that a positive Wassermann is particularly associated with macrocytosis.

21. The maternal deaths recorded were mostly amongst the macrocytic and normocytic cases.

22. Prematurity and early death of the child both show a high correlation with severe anæmia and macrocytosis. The neo-natal death rate in the anæmic classes is over 36 per cent amongst infants of mothers with macrocytic anæmia, 20 per cent amongst those with normocytic, and was *nil* amongst those in our series with microcytic anæmia. This is also evident amongst those cases first seen post-partum, for the neo-natal death rate was over 30 per cent in the combined macrocytic and normocytic groups and again *nil* in the microcytic.

23. In the post-partum cases there was evidence of a rapid decline in the influence of the macrocytic-anæmia-producing factor.

Conclusions.

Ætiology.—One of the objects in carrying out this inquiry was to obtain evidence of the important ætiological factors in the development of severe anæmia in pregnant women in Calcutta, and thus provide a rational basis for a campaign against the high anæmia mortality.

Considerable evidence was found in the whole series of cases of an *iron deficiency.* The hookworm infection rate was negligible amongst the patients whose stools were investigated, and, as it is not usually high amongst women of the class from which the patients were drawn, it seems probable that the origin of this iron-deficiency anæmia is dietary, i.e. the iron intake is insufficient for the extra requirements of the pregnant woman. The higher incidence of anæmia in the lower economic groups supports this suggestion and the fact that this 'poverty' anæmia is not predominantly microcytic is not necessarily against it, for it is probable that the microcytic picture of the anæmia is overshadowed by other influences.

A relative iron deficiency occurring in the child-bearing period of life with a tendency to exacerbations during pregnancy and lactation is well recognized in other countries, and is especially common amongst the poor whose iron intake is near or below the border-line of the body's requirements. In our series the pure microcytic anæmias occurred mainly in multiparæ and older age groups suggesting that iron depletion during earlier pregnancies may have been the cause.

Microcytic anæmia appeared to be associated with the observance of *purdah.* The pallor and debility which results from close confinement to the house and lack of fresh air and sunlight is probably similar to the chlorosis of a previous generation in England, which quickly responded to iron therapy.

This iron deficiency, however, though widespread, is of secondary importance in this series of cases of anæmia, for the majority, as well as most of the worst cases, were macrocytic. The far greater frequency of very severe anæmia amongst the macrocytic group may be evidence that the macrocytic-anæmia-producing factor is the dominant one.

The production of macrocytic anæmia appears to be influenced by several factors. In the first place, it appears to be connected in some way with the presence of the fœtus; the anæmia and the macrocytic tendency show a progressive increase throughout pregnancy, both in the anæmic patients and in the controls, and they tend to decrease immediately after parturition. Secondly, that this anæmia is *nutritional in origin, is suggested, not only by the higher incidence in the lower* economic classes, but by the fact that it is the macrocytic type that is more closely correlated with low economic status and that in the richer classes, vegetarianism, with its lower protein intake, is correlated positively with macrocytic anæmia. It is possible that the higher incidence of anæmia and macrocytosis in the second half of the year is connected with the less varied dietary, and with a lack of fish and vegetables, that is taken during the hot weather and monsoon months. Further, the common association of macrocytic anæmia with sore mouth, another deficiency condition, still further suggests a nutritional defect.

Thirdly, the association of severe macrocytic anæmia with primigravidity, independent of age, its occurrence relatively early in pregnancy, and its association with premature delivery and a high neo-natal death rate, suggest comparison with the toxæmias of pregnancy. This is further supported by the common association of macrocytic anæmia with œdema and albumin in the urine.

The significant positive correlation of severe macrocytic anæmia with enlarged spleen and liver, hyperbilirubinæmia and reticulocytosis all point to some hæmolytic condition and are especially suggestive of chronic malaria. Malarial parasites were recorded in only a few cases, but this is beside the point as it is not suggested that acute recently-acquired malaria is the cause, but chronic, or 'latent', malaria with hyper-reticulo-endotheliosis.

Another suggestion for an ætiologically associated infection is provided by the high correlation with a positive Wassermann reaction (with the possible proviso that this positive Wassermann reaction may be another manifestation of an association with malaria).

Finally, the occurrence of diarrhœa or a history of dysentery, often accompanied by prolonged adherence to a limited and unbalanced diet, suggest a state of gastro-intestinal dysfunction. This raises other ætiological possibilities for which added evidence is provided by the similarity of this type of anæmia to the macrocytic anæmia which occurs in association with sprue and cœliac disease.

To summarize, findings seem to suggest that the macrocytic anæmia of pregnancy is a 'conditioned' toxæmia, that is, a toxæmia associated with the presence of the fœtus and conditioned by a low dietary intake, or deficient absorption, of certain essential blood-forming and protecting substances, the syndrome being aggravated by (or perhaps only operative in the presence of) either a chronic malarial infection, with the associated hyper-reticulo-endotheliosis and excessive blood destruction which causes an extra demand for these blood-forming essentials, by chronic intestinal infections and diarrhœa with consequent exaggeration of mal-absorption, and/or by syphilis with its hæmopoietic depressing action.

Such a conditioned toxæmia is not purely hypothetical and has been shown to occur in connection with both lead and selenium poisoning and with sulphanilamide administration, in the former case it is recognized that people on a rich diet may escape the effects of lead poisoning, whilst others on a poor diet, suffer, and recently Smith *et al.* (1940-41) have shown that in animals a high protein diet will counteract the effect of chronic selenium poisoning and the hæmopoietic depressing action of sulphanilamide.

The fact that a macrocytic anæmia similar in many respects to the macrocytic of pregnancy occurs, although less commonly, in men in Calcutta, suggests that, as in the case of the iron-deficiency anæmia, the fœtus is the last straw that breaks the camel's back.

Clinical picture.—A further object in mind in carrying out this inquiry was to correlate clinical syndromes with different hæmatological groups, so that treatment in the absence of complete blood examination might be more rational. The findings of the inquiry have, however, in the first place impressed on the workers the complexity of the problem of the anæmias met with in Calcutta and the absence of any clearly-defined clinical types which can be correlated with the hæmatological groups according to which the cases were analysed. It is probable that one reason for this is, as has already been suggested, that an iron-deficiency condition may underlie other superimposed types of anæmia, leading in many instances to a confused hæmatological picture. Our findings do, however, emphasize the existence of marked clinical differences between cases of moderate anæmia (class B) as compared with severe anæmia (class A), and further, certain clinical distinctions can be drawn between the severe macrocytic anæmias, and the severe microcytic anæmias in class A.

The main differences between moderate and severe anæmias in general can be summarized as follows :—

In moderate anæmia (class B) the patient usually makes no complaints and the condition is found on routine examination. On inquiry she may admit to feeling tired, but many women expect this in pregnancy and think nothing of it. On examination some pallor of the mucous membranes of the mouth (and of the vagina, if the pelvis is healthy), is a more reliable guide to the degree of anæmia than the conjunctivæ or nails. The heart may be normal, but in the border-line cases will probably show a hæmic murmur or slight dilatation.

Post-partum hæmorrhage is rather more likely to occur in these moderate cases than in very severe anæmia.

In severe anæmia there may or may not be presenting symptoms. The degree of anæmia which may develop without complaints is a striking testimony to the low standard of well-being with which so many women seem satisfied. It is not uncommon to find a hæmoglobin of 30 to 40 per cent on routine examination. In more severe cases in this group, questioning will, however, elicit symptoms of increasing lassitude, or shortness of breath, palpitations and often swelling of the feet and face. Diarrhœa is sometimes the presenting symptom. On examination, marked pallor is seen, and the skin often has an 'alabaster' appearance

due to a combination of pallor and œdema. According to the degree of severity, the following signs will be found: œdema of feet, face or of the whole body, a degree of dilatation of the heart, a systolic murmur localized in the pulmonary area or audible all over the præcordium, the spleen may or may not be palpable.

Premature delivery, still birth and neo-natal deaths are common in this group. Tendency to post-partum hæmorrhage is *not* marked, sepsis in the puerperium is a danger.

The differences between the macrocytic and the microcytic cases in class A (severe anæmia) can be summarized as follows, but it must again be emphasized that the types are not by any means clear-cut.

Macrocytic anæmia has a seasonal incidence and cases tend to occur in the second half of the year. It is found on the whole rather more often in the first and second pregnancies irrespective of the age of the patient. These cases come under observation earlier in the course of pregnancy than the microcytic type and show a greater tendency to spontaneous recovery after parturition. They respond little, if at all, to iron therapy. Symptoms include a larger proportion of cases with a history of diarrhœa or dysentery than in the microcytic type. A yellowish tinge of the conjunctivæ and skin is often noticeable, corresponding to the large number in this group who show evidence of hyperbilirubinæmia. The mouth may be sore and the tongue be seen to have a shiny smooth appearance with red sore patches. It is usual to find enlargement of the spleen and liver. Labour is often premature, generally easy, and these cases do not often suffer from post-partum hæmorrhage, although delay in delivery of the placenta is not uncommon. The added strain of labour may be more than the enfeebled circulatory system can stand, and in extreme cases death occurs from heart failure during or soon after delivery. The neo-natal death rate is very high. A positive Wassermann reaction was more common in macrocytic than in microcytic cases in our series.

The severe *microcytic anæmias* show a positive correlation between Mohammedanism and purdah, the influence of the one perhaps overshadowing the other. Cases of this type of anæmia tend to come under observation later in the course of pregnancy than the previous type. Cases occur at a later age period and in the later pregnancies. The tendency for spontaneous recovery after delivery is less marked, but the reaction to iron both during pregnancy and afterwards is good. The conjunctivæ and mucous membranes of cases in this group are dead white rather than yellowish white, and signs of enlarged liver and spleen, sore mouth and intestinal complaints are less common, although by no means always absent. Heart failure in severe cases is equally possible, although in the series under investigation no microcytic case died; the explanation of this may be that severe microcytic cases were uncommon, for in the very severe cases both factors were probably operating and the macrocytic picture predominated.

The neo-natal death rate is low, and was actually *nil* in this series, for the infant has the first call on the available iron.

Recommendations regarding prophylaxis and treatment

It was no part of the programme of this investigation to assess the value of any particular form of treatment, and in fact we had very little opportunity to do so, for in the severe cases usually many different forms of treatment were given in quick succession or at the same time; our recommendations are therefore based on deductions from our hæmatological observations combined with other experience on our part and on that of others.

Prophylaxis.—It is encouraging to consider that no other fatal complication of pregnancy can, in theory, be so easily prevented if taken in time, as severe anæmia.

Prenatal care must include constant watchfulness for any signs of development of anæmic states. Regular examination of the woman for pallor of face and mucous membranes, and the rough estimation of the hæmoglobin by the Tallqvist method are a matter of a few moments' work, are within the capacity of any trained midwife to carry out, and will serve to detect all the grosser cases. The medical examination which should be undertaken in the early months of pregnancy should include a careful inquiry into the history of hæmorrhages, especially with reference to their frequency, of malaria, dysentery and syphilis, and of anæmia and œdema in previous pregnancies. The diet should be enquired into and any deficiency adjusted. Physical examination should be directed, especially towards the colour of the mucous membranes, the presence of a hæmic murmur or dilatation of the heart, the condition of the spleen and liver, œdema of feet, and the examination of the urine and blood pressure.

The pregnant woman's diet should ordinarily be the diet to which she is accustomed, with any necessary additions to make it 'balanced'. A pint of milk should be considered a minimum requirement, and this is more especially important in a vegetarian diet. Some substance rich in vitamin B complex (sprouting gram, rice polishings, marmite) should be taken, and articles containing a plentiful supply of salts including iron should be encouraged. Sunshine and fresh air should be insisted upon, and women who observe purdah should take extra vitamin D.

All pregnant women of the classes from which we drew our cases should be given iron by mouth as a prophylaxis during pregnancy. It is suggested that the first course should be given in the 3rd or 4th month and the second between the 6th and 8th month.

If a patient has an enlarged spleen or gives a history of malaria, a course of quinine may be advisable. This should be given after the third month is completed, and not at the time when menstruation would have occurred, and the patient should rest during the administration. The quinine should be given in 5-grain doses, three times a day combined with bromide. The chances of quinine causing abortion is exaggerated; the dangers to which the fœtus is subjected are far greater if quinine is withheld than if it is administered in moderate doses, but the time for its use should be carefully chosen, unless active malaria is present when in any circumstances thorough treatment must be given.

A Wassermann reaction should form part of a pre-natal examination and, if it is positive, treatment should be instituted.

If a history is obtained of severe anæmia in a previous pregnancy, a prophylactic course of iron by mouth, and liver by mouth or injection should be given early in pregnancy and blood examinations made at intervals throughout.

Diagnosis and treatment.—The first essential is accurate diagnosis and the detection of underlying and associated diseases. When anæmia is found, a full hæmatological examination should be carried out to discover the type of anæmia present, the stools and the urine should be examined, the blood pressure taken and a Wassermann test carried out.

The earlier treatment is instituted the more likely is the patient to respond.

Treatment in pregnancy.—The diet should be inquired into and suitably supplemented.

Sunlight and fresh air should be insisted upon.

If the anæmia is microcytic hypochromic, iron should be given in full doses, e.g. ferrous sulphate tablets, 6 grains, twice a day after food, iron and ammonium citrate, 40 grains, three times a day, or better still ferrous ammonium sulphate, 20 grains with glucose twice daily. All iron preparations should be given after food with a glass of water. Full doses of iron are not constipating, but diarrhœa and indigestion are sometimes complained of, and the pregnant woman is more particularly sensitive in this respect. The tablets and pills are more irritating than mixtures but the former keep better and are easier to administer. Iron 'plastules', a proprietary preparation that contains peppermint, are also convenient and will be found useful in cases in which the ordinary ferrous iron tablets cause gastric irritation.

If the anæmia is macrocytic and hyperchromic, liver extract should be given by mouth or injection. In pregnancy the time factor is important and injection is to be preferred.

If the anæmia is normocytic and orthochromic both liver and iron are indicated.

When there is failure to respond to treatment, the question of blood transfusion arises. Large transfusions are generally contra-indicated by the state of the circulatory system (œdema, etc.), but small transfusions may be given, or better still transfusions of moderate size by the drip-feed method. It is, however, doubtful whether evidence has been produced to show that these are any more effective than intramuscular injection of whole blood, which obviates grouping, is much easier to obtain, less distressing to give, and does not contain the risk of severe reactions, which seems particularly liable to occur in very anæmic women given intravenous transfusion.

Intramuscular injections, 20 c.cm. on alternate days, should certainly be tried in cases that fail to respond to other treatment: A reticulocytosis should be observed in about a week.

It should be remembered that anæmia will not respond to treatment in the presence of active sepsis.

(In milder cases of anæmia when facilities for blood examinations are not available, it is a good working rule to give a course of iron and observe the result; if there is no improvement, liver injections should be given in addition. If the skin shows a yellowish tinge, liver should be given from the first, and a careful inquiry for underlying conditions, e.g. malaria, syphilis, dysentery, carried out.)

During labour.—Many cases of severe anæmia come under observation for the first time when labour has begun.

In very severe cases the patient is suffering from a degree of heart failure and anoxæmia, and the strain of labour through which she has to pass may well be too much for her cardiac reserve. Labour in these cases is, however, often premature and generally easy (*cf.* labour in cardiac disease, with which these cases have much in common), and it is surprising how women with red-cell counts of a million, or even just under a million, may survive.

A minimum of excitement and disturbance of the patient is necessary, and delivery should be allowed to take place in a quiet room, preferably on a bed, with the patient half sitting, well propped up on pillows, or with a back rest. Frequent *small* feeds with plenty of glucose should be given. Sedatives usually helpful are paraldehyde, bromide and chloral. Morphia may be needed, and has the added advantage of preventing the tendency to restlessness which is induced by the cardiac distress and anoxæmia. It may have to be combined with atropine if there is œdema of the lungs.

The œdema and disturbance of the circulatory system contra-indicate the use of large transfusions of blood or subcutaneous injections of fluid, but small intravenous injections of glucose may be used to tide the patient over a critical period.

We have observed no good results from hypodermic injections of strychnine and digitalin, but camphor and coramine sometimes seem to help. On the whole, sedatives are indicated rather than stimulants.

The third stage should never be hurried. Efforts to express the placenta are particularly exhausting to the patient and should never be made. Post-partum hæmorrhage is rare in severe anæmia but particularly dangerous if it does occur. Spontaneous expulsion of the placenta nearly always occurs unless death is imminent.

Treatment in the puerperium.—If the patient with very severe anæmia survives the first few days after delivery she nearly always recovers, unless sepsis supervenes. The danger of puerperal infection is however very great, and in some conditions of practice, it is probably true that as many very anæmic women die later from puerperal sepsis as die from the effects of anæmia and the strain of labour. When sepsis occurs the difficult question arises of the use of the sulphonamides with their depressant effect on the hæmopoietic system. But as the anæmia will not improve in the presence of the marked sepsis, there is no alternative but to give them combined with iron, liver and intramuscular injections of whole blood, or continuous blood transfusion when circumstance permit, at the same time.

APPENDIX II

Recommendations based on the findings in 40 deaths from anæmia in pregnancy in Delhi and Najafgarh during a 12-month period (1938-39). From a report of the Delhi Maternal Mortality Inquiry (Indian Research Fund Association) by M. I. Neal Edwards, M.D., W.M.S., and J. Dei, M.B., B.S.

Recommendations

1. *Anæmia: need for emphasis in medical education and training of midwives*

(*a*) Anæmia is, next to puerperal sepsis, the most frequent cause of maternal death in India. Therefore in the training of medical students and pupil midwives particular emphasis should be laid on its prevention, detection and treatment. Many textbooks in current use give no more than a passing reference to the subject. This defect needs to be corrected, and especial emphasis should be laid on anæmia in lectures and demonstrations.

(*b*) A method of hæmoglobin estimation should be taught to every midwife, and the means of carrying it out (e.g. Tallqvist scale) should be considered part of her equipment. The use of this rough method, although not providing very much information, does serve to impress the matter of anæmia on patient, doctor and midwife alike, and for this reason has a more than intrinsic value.

2. *Prenatal care: detection of anæmia*

(*a*) Regular examinations of the pregnant woman for the signs of anæmia should be the rule. A search for pallor of the face and mucous membranes, and the rough estimation of the hæmoglobin by the Tallqvist method, are a matter of a few moments' work, and will serve to detect the grosser cases. Regular hæmoglobin estimation in pregnancy should be considered as essential a part of prenatal care as the examination of the urine for albumin. (It is probably true to say that were it done regularly in India, more lives would be saved thereby than are saved at present by urine examination.)

(*b*) A knowledge of the type of anæmia and the response to treatment is necessary for rational and successful therapy, therefore arrangements should be made for doing the simpler blood examinations in connection with prenatal clinics

and women's out-patients departments. But the more advanced hæmatological technique is very specialized and therefore special arrangements, such as a central laboratory, should be made where full investigations can be done.

3. *Treatment*

(*a*) Welfare centres and women's hospitals should carry large supplies of iron, liver and vitamin-B preparations, for use in the treatment of anæmia.

(*b*) Prenatal and lying-in wards for anæmia cases should be as important a part of maternity hospitals in India as wards for puerperal sepsis. When the frequency and danger of this complication of pregnancy are better recognized by the medical profession and the public, and more cases apply in time for admission to hospitals, separate wards for the purpose of treatment will be a necessity.

4. *Importance of diet in prevention of anæmia*

(*a*) More attention should be directed to the giving of a sufficient and balanced diet during illness and convalescence. Anæmia often occurs after periods of partial starvation and defective nutrition, especially when this is associated with chronic gastro-intestinal disorders, when a low diet, poor appetite and defective absorption, all contribute towards its development. This is even more likely to happen during pregnancy when the fœtus is a constant drain on the mother's resources. Much could be done to prevent anæmia by more attention to the convalescent patient. Few women leave maternity hospitals in India who would not be the better for diet advice and a course of intensive treatment with iron; by means such as this, which might be made routine, much gross anæmia in women could be prevented.

(*b*) The possible association between a vegetarian diet and macrocytic anæmia needs investigation, and the matter should be studied in different localities.

APPENDIX III

Propaganda leaflets

A. Anæmia in pregnancy*

The dangers of anæmia

Anæmia means lack of blood. An anæmic person looks pale and feels weak. There may be no other symptoms, but the condition leads to much suffering and is a special danger during pregnancy, when it often becomes rapidly worse. Many people in Calcutta are anæmic and as a result are less energetic and healthy than they might be.

In pregnancy

Anæmia begins insidiously with increasing pallor of the skin and shortness of breath. The mother feels tired, has loss of appetite and fails to digest and enjoy her food. She may think that this is due to pregnancy only, but it is not so; in pregnancy a woman should feel quite well. Later she may become unable to carry on her household work because of the anæmia, and she feels the strain of the growing child to be increasingly great as pregnancy advances. Later still, great weakness and swelling of the feet and other parts of the body may occur and this means that the heart has been affected.

The child as it develops is weak also, it may be born prematurely, or even be stillborn, and if it is born alive it is often too weakly to survive for long.

During confinement

The strain of labour for an anæmic mother is very great. Instead of having an easy delivery resulting in complete restoration to health in a short time, the mother remains pale and feeble, she often has very little milk for her baby, and she remains an invalid for many months. It may happen that she cannot bear the strain of labour, or some complication may arise because of her anæmia, and she may lose her life.

These suffering mothers bring great trouble and sadness to their families, but this can be avoided by proper care and it is in the hands of the educated members of the family, the fathers and husbands to take steps to prevent the mothers' suffering and ensure their health and safety during pregnancy and childbirth.

* Prepared for Calcutta Health Week Exhibition, 1937.

How to avoid anæmia

There are many forms of anæmia which arise from different causes :—

(1) *Certain diseases*, e.g. malaria, may cause anæmia. The cure for this kind of anæmia is proper treatment of the cause.

(2) Other kinds of anæmia come from *lack of fresh air and sunlight*. Just as a plant grown in the dark becomes weak and pale, so does a woman who is shut up inside the house for long hours become anæmic. It is most important that purdah women should take mild exercise and sit out of doors in the morning and evening.

(3) *A faulty diet* may cause anæmia if it consists of foods which do not contain sufficient iron. This commonly happens in pregnancy, for at this time the mother needs iron for the growing child as well as for herself. A pregnant woman should eat a plentiful supply of fruit and green vegetables. Tomatoes, carrots and spinach are especially good. Eggs and meat also are foods rich in iron.

(4) *Pregnancy* itself may cause anæmia in women. This can be avoided by taking liver in soup or curry, or if this kind of food is not eaten, taking a tablespoonful of marmite or yeast every day.

B. Anæmia*

Anæmia means that the blood is of poor quality. Good health is largely dependent on good blood. If the blood is poor the person looks pale and feels weak and easily falls a prey to illness.

Anæmia may be caused by loss of blood, or it may be the result of chronic illness, or it may be due to faulty diet. Sometimes it develops rapidly in pregnancy and is then a very dangerous condition for mother and child.

To prevent anæmia

1. Take regular exercise in the fresh air and sit or walk in the sunlight for at least half an hour every day.

2. Keep the house well ventilated and sleep with windows open at night.

3. Take good, ordinary diet and see that it includes plenty of green vegetables, e.g. spinach, and fruit, e.g. tomatoes, apples, grapes, pomegranates.

Non-vegetarians should take liver, meat and eggs.

To cure anæmia

1. A doctor should be consulted at once if signs of anæmia are present because a mild form may quickly develop into a dangerous condition especially in pregnancy.

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* Published by the Maternity and Child Welfare Bureau of the Indian Red Cross Society, New Delhi, in Hindi, Urdu, Bengali and English. Price 8 as. per 100.

The doctor will examine the blood and prescribe the treatment required.

2. The diet is of great importance in anæmia therefore a good diet must be taken. *In addition to ordinary food take every day:—*

Carrots and spinach and other green vegetables; tomatoes and fruits in season such as apples, grapes, oranges.

Non-vegetarians should take liver at least twice a week and plenty of meat and eggs.

Vegetarians should take marmite: two teaspoonfuls thrice daily.

Note.—If there is any chronic disease present, e.g. hookworm, dysentery, pyorrhœa, it is necessary to get this treated if the anæmia is to be cured.

How to cure anæmia

When there is anæmia in pregnancy immediate steps should be taken to get it properly treated, or it will certainly get worse as pregnancy advances.

(1) *A doctor* should be consulted who will examine the blood to find out what kind of anæmia is present.

(2) *Treatment* given by the doctor will be directed towards removal of the cause, and if necessary alteration of the diet and change of daily habits as regards sunshine and fresh air. Iron medicine and injections of a liver preparation will be prescribed and marmite will be recommended.

If the anæmia is extreme it may be necessary for the pregnant mother to b admitted to hospital for intensive treatment which may have to include transfusior of blood.

All women should be under the care of a doctor, welfare centre or hospita antenatal department during pregnancy, and blood examinations should be ma in order that anæmia if present may be detected. Only in this way will an be treated in the earliest stages when it can be easily cured and the mother a child saved from its dangers.